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STUDIES OF THE CHOLINESTERASE ACTIVITY OF THE AQUEOUS HUMOUR IN MAN AND SOME ANIMALS

WITH SPECIAL REFERENCE TO THE INFLUENCE OF CERTAIN ANTICHOLINESTERASES ON IT

BY

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PREFACE

The subject of this investigation was suggested to me by Professor Armas Vartiainen, M.D., who also kindly placed at my disposal the laboratory facilities and the experimental animals of the Department of Pharmacology in the University of Helsinki. Experimental work was started in the autumn of 1951 and completed in 1953. I am greatly indebted to Professor Vartiainen for supervising and guiding my work with encouraging interest and invaluable advice through all phases of the investigation.

I am grateful to Professor Mauno Vannas, M.D., head of the Ophthalmological Clinic of the University of Helsinki, for his kindness in allowing me to obtain the samples of human aqueous humour necessary for the work.

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Certain analytically pure anticholinesterases were kindly presented by Bo Holmstedt, M.D., Stockholm, and other preparations were received from the pharmaceutical manufacturers Bayer, Leverkusen; The British Drug Houses, Ltd., London; F. Hoffmann-La Roche, Basel; and E. Merck, Darmstadt. The kindness of T. Litko, V.M.D., director of the slaughterhouse of the Karjakunta Meat Co-operative Company, Helsinki, and the ready assistance of his workers enabled me to collect samples from slaughter animals. To all these persons and corporations I extend my appreciate thanks.

I am also grateful to Mrs. Kersti Lampen, who most willingly assisted me at all times in the drawing of samples.

This report has been translated into English by Miss Elvi Kaukokallio.

Helsinki, April 1954

Kaisu Viikari

CONTENTS

LIST OF ABBREVIATIONS

a.ch.	anterior chamber
ACh	acetylcholine
AntiChE	anticholinesterase
aq.h.	aqueous humour
BCh	benzoylcholine
ChE	cholinesterase
DFP	diisopropyl fluorophosphate
HETP	hexaethyl tetraphosphate
LD	lethal dose
MCh	acetyl-β-methylcholine
Mintacol	p-nitrophenyl diethylphosphate
Tabun	dimethylamido-ethoxyphosphoryl cyanide
TEPP	tetraethyl pyrophosphate

INTRODUCTION

The active research work instigated by World War II produced a large group of substances for use as war gases and very injurious to the living organism because of their inhibitive action on cholinesterases. Substances in this group are organic derivatives of phosphoric acids and so far their greatest benefit to mankind has probably been in the form of insecticides. However, experiments have already been made with some of these substances with a view to their application to the service of medicine, and they even have been used successfully in the treatment of, for instance, glaucoma and myasthenia gravis.

Polyalkylphosphates differ in many respects from the previously known anticholinesterases, physostigmine and neostigmine. Most of them inactivate cholinesterase irreversibly and abolish rapidly the effect of atropin. A relatively frequent observation has been an increase in intraocular tension, instead of a decrease, which occurs more regularly with physostigmine and neostigmine.

A number of questions are therefore evoked concerning the effects and the mechanism of action of these substances. Since they are anticholinesterases their effect on the cholinesterase of the eye is of great interest. Aqueous humour is a perfusion fluid of the eye and is obtained relatively easily for purposes of investigation. It was therefore tempting to set out to seek an answer to the questions regarding the effect of anticholinesterases on the cholinesterase activity of the aqueous humour.

PART I: CHOLINESTERASE IN THE AQUEOUS HUMOUR

EARLIER INVESTIGATIONS

The concept of cholinesterase (ChE) is not old, but it is closely related to the history of physostigmine (eserine) and acetylcholine (ACh). Although physostigmine was known already since 1863 (Fraser) as a poisonous alkaloid present in the Calabar bean and Laquer demonstrated in 1876 its effect on the increased pressure in the glaucomatous eye, the mechanism of its action was not made clear until at a much later date.

As a synthetic substance ACh has been known since 1867, but its physiological significance was not known until 1906 (Hunt and Taveau), and because of its rapid destruction in the blood Dale (1914) suggested that some kind of an esterase was present in the latter. Abderhalden and Paffrath (1925) were able to demonstrate such an enzyme in the intestine of the pig and the horse. Knowledge on the physiological significance of ACh began to be obtained when Loewi (1921) observed that some substance is released during vagus stimulation. This formed the basis for the now universally known theory of the neurohumoral transmission of nerve impulses. At that time, however, the nature of the substance in question was not known more closely, and it was only several years later that Loewi and Navratil (1926 a) reported their assumption that the »Vagusstoff» is identical with ACh. At the same time they observed that its rapid inactivation is due to the enzymic decomposition of ACh and that physostigmine inhibits the destruction of ACh by the esterase in the frog heart (1926 b). Stedman, Stedman and Easson (1932) called this enzyme »choline-esterase». The ACh—ChE system has since then continued to be a subject of particular interest to

investigators. As late as in 1928 Galehr and Plattner were still pondering upon the question that ACh and the »Vagusstoff» are probably identical. In the following year Dale and Dudley (1929) were successful in isolating ACh from ox spleen.

A notable proportion of the voluminous literature on this matter deals with ophthalmic aspects, a far from negligible number of studies having been made on the ACh and ChE activities in ocular fluids and tissues. However, opinions on the ChE activity of aq.h. frequently are conflicting. These contradictory results should probably be ascribed chiefly to the use of methods of greatly varying sensitivity.

Passow was the first to suspect, in 1930, a possible correlation between ACh and glaucoma, having observed after instillation of ACh into the glaucomatous eye a definite, although transient, fall in the intraocular tension, without notable change in the size of the pupil.

In the same year Plattner and Hintner (1930 a, b) studied the tissue extracts and fluids of a number of mammals. They found that the aq.h. of the rabbit (the amount withdrawn is not stated) hydrolysed a minute amount of ACh, whereas human urine and certain other fluids gave entirely negative results. The titration was carried out on frog heart with a sensitivity to ACh of 1 : 400—500 million, and physostigmine was found to completely inhibit the hydrolysis of ACh by aq.h.

The theory of the presence of ChE in aq.h. was confirmed in studies carried out by Engelhart in 1931. After instillation of physostigmine into the rabbit's eyes, they were exposed to light to allow it to stimulate the parasympathetic nerves and the aq.h. was then tested on frog heart, which brought out ACh activity.

A similar test method was employed by Luco and Lissak in 1938 but the results which they were able to obtain with rabbits were too conflicting to permit the drawing of any conclusions. On the other hand, they were able to demonstrate with cats that light stimulus produced ACh in the aq.h. in an amount approximately corresponding to a dilution of 1 : 100 million when tested on frog heart. The amount of 0.2 cc of aq.h. obtained from rabbits was only sufficient for one test on frog heart, whereas 1 cc withdrawn from cats permitted the carrying out of three tests. No ACh was demonstrable in the physostigmine-treated eye not exposed to light.

Velhagen (1930) injected subconjuctivally into cats aq.h. drawn from the cat eye after faradic stimulation and observed a contraction of the pupil. The same aq.h. also clumped together the melanophores in the iris; this, as well as contraction of the pupil, is known to be characteristics of ACh. Using extracts of bovine uvea and retina on cats, Velhagen (1932) effected a decrease in the blood pressure, which is very sensitive to ACh. This effect was not seen when aq.h. was added to the extracts. However, a small amount of physostigmine was in its turn capable of inhibiting the action of aq.h. Velhagen interpreted this series of tests as evidence that the extracts in question contained ACh, whose diminishing action on the blood pressure was inhibited by the ChE in aq.h., and that the activity of the latter was inhibited in turn by physostigmine.

Uvnäs and Wolff (1938) carried out ChE measurements in bovine aq.h. by the Pulfrich colorimetric method but found practically no ChE except 5—6 hrs. *post mortem* and in partially re-formed secondary aq.h. They assumed that the ChE demonstrated by them originated from the vitreous body. The amount of aq.h. drawn by puncture was 0.5 ml.

Pletneva, Raewa and Voronina (1938) studied the biological effect of human aq.h. on the frog heart. The aq.h. of 34.5 per cent of glaucomatous patients showed effects similar to those of ACh, whereas the biological analysis of the aq.h. of the patients with cataract indicated no changes. Using bovine aq.h. on the leech, Leidig (1938) failed to obtain an ACh reaction.

Weve and Fischer (1939) reached a totally negative result in colorimetric studies of the ChE activity of human, bovine and horse aq.h. They stressed that the utmost care should be exercised in drawing aq.h. and that it should not be aspirated.

Brückner (1943 a), using Ammon's method, regularly found ChE in the aq.h. of cows and horses killed ¼—5 min. previously; in fact, there was even more ChE in relation to the amount of protein in aq.h. than in the blood. Since a sample taken from the same eye 40 min. later showed only a slight increase of ChE, no consideration need be given, in his opinion, to possible sources of error (compression damage to eyeball, diffusion from the vitreous body, and postmortal changes).

In ChE determinations made in the same year from different

parts of bovine uvea, Brückner (1943 b) confirmed the opinion that ChE is accumulated at the sites where ACh is produced.

Bloomfield (1947) studied in the manner of Engelhart the effect exerted on the frog heart by ACh in the aq.h. drawn from glaucomatous and non-glaucomatous eyes following physostigmine instillation and light stimulation. Under 4 per cent procaine hydrochloride anaesthesia, 0.1 cc of aq.h. was aspirated (from the a.ch.) and added undiluted to the cannula. The aq.h. from non-glaucomatous eyes gave a reaction corresponding to an ACh concentration of 1 : 100 million, whereas no reaction was obtained in 15 out of 20 glaucomatous cases and the remaining 5 cases yielded an ACh reaction below normal. Successful filtering operations also failed to alter the ACh content. The ACh reaction was inhibited by 0.1 cc atropine diluted 1 : 10,000. In 2 non-glaucomatous cases the ACh activity corresponded to a concentration of 1 : 1 million and caused the frog heart to stop on the diastole.

The observations made by Bloomfield brought up the question whether ACh deficiency in aq.h. is an etiological factor in glaucoma or a results of the disease.

Matteucci (1947) made comparisons of the ChE values in the aq.h. and blood of patients with glaucoma, using the Warburg-Barcroft technique. To prevent the admixture of vitreous body he permitted, in the manner of Amsler, only an amount of 0.05—0.02 cc of aq.h. to flow without aspiration. *He found no ChE in the aq.h. of the normal eye.* The glaucomatous aq.h. gave CO_2 values of 0—(0.1—) 0.8 cu mm, which indicates ChE activity. The serum ChE levels usually were also above normal, as had been found by Gallois and Herschberg (1946). No deviations from the normal blood ChE values were found by Rados (1943) in his series of 61 patients with glaucoma.

Thomas *et al.* (1947) observed no ChE activity in aq.h. from glaucomatous eyes. Simultaneous tests with the sera of the same patients gave variable ChE values.

Jaffe (1948) arrived on theoretic grounds at the conclusion that since ChE is a protein and the protein content of aq.h. is almost nil, there should be at best only faint traces of ChE in the aq.h. He made ChE determinations of the aq.h. of the cat by a modified Stedman gasometric technique (Stedman and Stedman 1935), employing Barcroft's apparatus. Less than 1 cc (at the most

0.85 cc) of aq.h. was removed by puncture, the complete emptying of the a.ch. thus being avoided. There were either no, or insignificant traces of ChE. On the other hand, the first and second aq.h. drawn after paracentesis definitely contained ChE in amounts which parallelled closely the increased protein levels in the plasma and aq.h. Jaffe stated that the protein content of the aq.h. has never been known to be increased in glaucoma and consequently ChE cannot be expected to be found in it.

However, contrary to Jaffe's supposition, de Grósz and Goreczky (1948) demonstrated an increased ChE activity in aq.h. from glaucomatous patients in the presence of normal serum ChE values. They employed the Amsler aspiration technique and regarded their results as an indication of local disturbance in the ACh metabolism, since the serum ChE levels were normal (Rados 1943). Later de Grósz (1950) estimated the ChE content of the glaucomatous eye at three times that of the normal eye. He stated that his results were in agreement with those of Bloomfield, who found no ACh production in advanced cases of glaucoma, in contradistinction to the normal eye.

In the opinion of de Roetth (1950), many investigators have made a basic error in endeavouring to measure ACh instead of the enzyme, as the former is rapidly hydrolysed. He maintained that only a few of the earlier papers stand up to present day criticism with reference to important general conditions such as enzyme and substrate concentration, optimum pH, and temperature range. In his own study de Roetth used Hestrin's colorimetric method, the sensitivity of which is about the same as that of Warburg's manometric method. No ChE was demonstrated in the aq.h. from 50 rabbit and 15 cat eyes, whereas in ox and horse eyes there were 0.56 and 0.16 ChE units, respectively. Differences in the rapidity and mechanism of reactions of the pupil have been cited in an effort to find a theoretic explanation for the variations in ChE activity in the different species of animals. He assumed that the ChE finds its way into the aq.h. from degenerated cells of the iris, ciliary body and retina or from the serum, but in view of its minute amount he presumed it has no functional significance, contrary to the opinion of many of the earlier workers. On the whole, he considered it useless to deal with the aq.h. in studying the ChE of the human eye, as aq.h. contains ChE in minute amounts that

are difficult to compare. Comparisons made by de Roetth of the ChE activity of the iris and the ciliary body of normal and glaucomatous eyes gave lower values for the glaucomatous eyes.

Experiments to classify the ChE in the aq.h. by types were also made by de Roetth. The aq.h. of the ox contained specific ChE. In the cat, ChE was demonstrable only in the secondary aqueous, in which, as well as in the cat's serum, it was non-specific. He regarded this as a confirmation of the assumption that ChE in the aq.h. of the cat comes from the serum.

Arató and Arató (1952), employing Zeller's modification of Ammon's method, studied the ChE activity of the serum and aq.h. of glaucomatous and non-glaucomatous human subjects. The ChE activity in the serum of patients with glaucoma was definitely lower than in normal serum but no ChE activity could be demonstrated in the aq.h. of normal or glaucomatous eyes. However, the differences observed in the blood permitted no inference as to the vegetative equilibrium of the eye, but they stressed that the ACh deficiency in the glaucomatous aq.h. is not due to increased ChE activity.

In contrast to the recent chemical methods, Toda (1952) made biologic determinations of the ChE activity of rabbit aq.h. and serum by means of the frog's m.rectus abdominis. ChE was demonstrated in the aq.h. in rare cases only, and the ChE activity of secondary aq.h. was practically similar to, but weaker than that of the serum. It was most marked 15—30 min. after paracentesis and very slight already at 4 hrs. Toda also held the opinion that the ChE of secondary aq.h. comes from the serum.

THE PROBLEMS

As was seen, the results of previous studies on the cholinesterase activity of aqueous humour are contradictory or, when the results are similar, the quantitative findings are at variance. An attempt has therefore been made in this investigation to find answers to the following questions:

Does the aqueous humour of the rabbit and certain other mammals including man contain cholinesterase, and if so, in what amounts?

Is it possible to determine whether such cholinesterase, if found, is specific or non-specific?

If cholinesterase is present, do anticholinesterases produce changes in the cholinesterase activity of the aqueous humour?

Studies of the cholinesterase activity of aqueous humours have usually been connected with investigations of the possible rôle of cholinesterase activity in the etiology of glaucoma. It is not the intention of the present study to deal with this very extensive subject.

SOME PROPERTIES OF CHOLINESTERASE

Cholinesterase (ChE) is an enzyme which acts as a catalyst in the hydrolysis of acetylcholine (ACh) into choline and acetic acid. When Stedman, Stedman and Easson in 1932 suggested for this enzyme the name »choline-esterase», they were not yet able to determine whether there exist one or more types of ChE. However, they expressed a suspicion that several types may be present. This opinion was confirmed in the following few years, when it was demonstrated that many sera contain at least two types of ChE and that physostigmine even in small amounts inhibits at least one of these types (Stedman, Stedman and White 1933, Shaw 1935, Easson and Stedman 1937).

Vahlqvist (1935) was the first to observe that human plasma does not hydrolyse choline esters only. In 1939 Gilman, Carlson and Goodman first spoke of »specific» ChE, which was almost completely inactivated by minute amounts of physostigmine, and of »non-specific» ChE, which was but little affected even by high concentrations of physostigmine.

An essential new feature was discovered in 1940 by Alles and Hawes, who studied human blood by chemical methods and found that the serum ChE and the erythrocyte ChE are not identical. A striking qualitative difference between the enzymes of human serum and of erythrocytes was seen in their relative actions upon ACh, as the erythrocyte ChE showed the maximum activity in low concentrations of ACh. The erythrocytes split acetyl-β-methylcholine (MCh), at the same rate as ACh, whereas the latter was only slightly hydrolysed by the serum.

Differences in the properties of different ChE enzymes became an important subject of investigation, and Mendel and Rudney (1943 a), among others, confirmed the above observations by demonstrating that the ChE of brain and red cells is specific and that of blood serum is non-specific and catalyses the hydrolysis of non-choline esters also. Their tests, like those of Alles and Hawes (1940), showed that the specific ChE exerts its maximum activity in low concentrations of the substrate, contrary to the behaviour of the non-specific ChE. The term »pseudo-ChE» proposed by them for the non-specific enzyme has not gained wide favour. A further means of differentiating the types of ChE was provided when Mendel, Mundell and Rudney (1943) reported that only the »true ChE» hydrolyses acetyl-β-methylcholine, whereas only the »pseudo-ChE» splits benzoylcholine (BCh).

Since then a large number of investigations have been carried out on the types and amounts of ChE in the tissues of various species of animals. With a few exceptions, the results may be classified into two groups. Tissues containing chiefly specific ChE include erythrocytes, nerve tissues (Nachmansohn and Rothenberg 1944, 1945) including the retina (Francis 1953), and thymus (Mendel and Rudney 1943 a, b), whereas blood serum, pancreas and glands (Strelizt 1944) contain non-specific ChE.

The studies of Hawkins and Gunter (1946) indicated that non-specific ChE plays no essential part in the hydrolysis of ACh *in vivo*.

The different types of ChE were also studied by Zeller and Bisseger (1943), who suggested the term »e-type» for the specific ChE of brain and erythrocytes, and »s-type» for the non-specific ChE of blood serum. They also called attention to the difference in the linkage mechanism. The reaction rate of the erythrocyte ChE decreased as the ACh concentration increased from a given amount, whereas the maximum activity of the non-specific ChE at high substrate concentrations was confirmed in their studies also.

The electrophoretic investigations of Augustinsson (1944, 1945) showed in the ChE types certain differences that are also chemically recognisable. Erythrocyte ChE seemed considerably more sensitive to changes in pH than serum ChE. In the following year (1946) he studied the differences by means of MCh and BCh. He has also compared in great detail the ChE activities of various animal

tissues by chemical methods and made a general classification of these enzymes. Using various esters he studied especially the enzyme activities as function of substrate concentrations. The results of Mendel and Rudney (1943 a) on the optimum substrate concentrations of various ChE:s proved erroneous because of an inadequate technique as the tests had not been carried out under optimum conditions.

Augustinsson and Nachmansohn (1949 b), like Stedman, Stedman and Easson (1932), suggested that the enzymes of serum and pancreas which hydrolyse choline esters be termed cholinesterases, as they hydrolyse choline esters at a higher rate than non-choline esters and as their physiological substrate is still unknown.

According to Augustinsson (1949) the erythrocyte ChE gives a rather sharp curve for optimum ACh concentration. Serum ChE gives the familiar dissociation curve. These dissimilarities serve to indicate the significance of the substrate concentration in determining ChE activity.

ChE is an enzyme which is generally present in living tissue and, like all enzymes, is a simple or compound protein (Bodansky 1938). It is probable that ChE is present in all or most tissues and that several types of ChE exist but only one type usually dominates. It is large-molecular, as it does not pass through a dialysis membrane. Opinions differ as to the protein fraction with which ChE is associated. According to Glick, Glaubach and Moore (1942), it is associated with the α- and β-globulin fractions but Faber (1943) and Augustinsson (1944) believe it to be closely related to the albumin fraction.

The study of ChE is greatly facilitated by the fairly high stability of the substance. It was observed already by Vahlqvist (1935) that plasma ChE stored in a stoppered test tube at room temperature will retain its activity for at least one week, and by Stedman, Stedman and Easson (1932) that the activity persists for several months if the serum is sterile. ChE is stable particularly in glycerine extracts; in fact, the activity has been found to increase with time in stored blood, probably due to the slow passage of ChE from the red cells into the plasma.

Effect of Temperature on Cholinesterase. — Destruction of ChE begins at +56° C (Loewi and Navratil 1926 a, Plattner and Hintner

1930 b) and the activity is quite lost at +70 to +75°C (Abderhalden and Paffrath 1925, Kahane and Lévy 1936). Active dry powder is obtained by evaporation of blood and tissue extracts (Bernheim and Bernheim 1936) but drying with acetone destroys the activity (Nachmansohn and Lederer 1939). ChE activity is not impaired by the freezing-drying process (Anfinsen, Lowry, and Hastings 1942, Augustinsson 1948). The optimum temperature for ChE activity of serum and nerve tissue is +37 to +40° C (*e.g.*, Kahane and Lévy 1936, Genuit and Labenz 1941).

Effect of Hydrogen Ion Concentration on Cholinesterase. — The specific ChE is considerably more sensitive to changes in pH than the non-specific (Augustinsson 1944, 1945). The optimum pH range for the specific enzyme is 7.5—8.0 (Alles and Hawes 1940), the activity is completely lost at pH 4.5, and even on the alkaline side it begins to decline earlier than that of the non-specific ChE (Augustinsson 1945).

The activity of the non-specific ChE is not destroyed before pH 2 and on the alkaline side the decline in activity begins only at pH 11 (Werle and Uebelmann 1938, Augustinsson 1945). The optimum pH for this enzyme is 8.0—8.5 (Bernheim and Bernheim 1936, Glick 1937, Werle and Uebelmann 1938).

The isoelectric points [e-ChE 4.65—4.70 and s-ChE 4.36 (Augustinsson 1944)] indicate a close relationship between ChE and albumins.

Inhibitors of Cholinesterase. — ChE is an enzyme of the animal organism. It would therefore seem probable that there also exists an antibody, an anticholinesterase (AntiChE), produced by the organism. However, such a substance has not yet been demonstrated. On the other hand, there are a large number of chemical substances which to a greater or lesser degree are capable of inactivating ChE. The most classical AntiChE is physostigmine, or eserine, which maintained its position of »first-born» for tens of years until Stedman in 1931 developed a synthetic antibody, neostigmine (Sollmann 1949). This substance is very similar to physostigmine in its effects. The marked inactivating action of physostigmine on ChE in concentrations of 1 : 1—40 million was already observed by Loewi and Navratil (1926 b), Engelhart and Loewi

(1930) and Matthes (1930). According to Augustinsson (1948), physostigmine in a dilution of 3.63×10^{-6} M inhibited strongly the activity of both specific and non-specific ChE at all substrate concentrations. Of the substances earlier known, methylene blue and certain anaesthetics (novocaine, pantocaine) had a marked AntiChE action and urethane also to some degree. Polyalkylphosphates, the new AntiChE substances introduced in recent years, differ from the earlier ones both in quantitative effect and mechanism of action. This subject will be discussed in more detail in Part II of this investigation.

DETERMINATION OF CHOLINESTERASE ACTIVITY

There are several methods for determination of ChE activity but they may be classified chiefly under two types, *viz.*, biological and chemical. The biological methods have been known for a long time but more recently the chemical methods have gained wider use, especially in large serial experiments (Ammon 1934, Oka 1954). However, the greatest drawback in the chemical methods is their low sensitivity, which is far below that of the biological methods. The latter, for their part, have the disadvantages of numerous technical difficulties and especially of the lengthy time required for the tests. However, many workers have employed the biological methods, for compared with the chemical procedures their sensitivity in the determination of minute amounts of ACh is 100—1,000 and even up to 10,000 times greater. Amounts down to 0.01 μg may be demonstrated by the biological methods, whereas the widely used Warburg manometric method and the Hestrin colorimetric method have been reported to be limited to ACh amounts above 50 μg (de Roetth 1950). The biological procedures thus offer possibilities to study the esterase under physiological relationships of substrate and enzyme concentrations. High enzyme and low substrate concentrations are recommended in these methods, while an inverse relationship is employed in the chemical methods. Because of these differences in the principle of the tests, the results obtained are not comparable (Mahal 1938). Even when biological methods alone are used for estimation of relative ChE amounts in various fluids, the dissimilar substrate—enzyme relationships may be a source of error.

Biological Methods. — Since it was obvious already in advance that one of the difficulties in the present work would be the availa-

bility of very limited amounts of aq.h. and presumably also of the ChE to be sought, biological methods alone seemed practicable.

The basic principle of the procedure is a simple and clear-cut one. The essential point on which the investigation is based is the estimation of the activity of ACh or other choline esters. For this purpose frog rectus, leech dorsal muscle, frog heart, mouse, rat and rabbit intestine, isolated rabbit heart auricle, certain less suitable organs (Webb 1950 a, b, Welsh 1950) and cat's blood pressure are usually employed. The action of ACh on these is manifested as muscle contraction, altered amplitude of the heart beat, or decreased blood pressure. The enzyme is allowed to act on a known amount of ACh under given test conditions, after which the residual amount of ACh is biologically titrated by comparing the reaction with that obtained with a known standard dilution of ACh.

PRESENT INVESTIGATION

METHODS

Use of the Frog M. Rectus Abdominis. — The greater part of the tests in this investigation were carried out with the m. rectus abdominis of the frog (*Rana temporaria*), which probably is the most widely employed test object for this purpose. Riesser (1921) was the first to describe the effect of ACh on muscle, the frog gastrocnemius, but use of the abdominal rectus muscle of the frog in the testing of tissue extracts was described in detail by Chang and Gaddum (1933) and Kahlson (1934 b). The procedure is as follows: The rectus muscles are dissected out and suspended from Gimbal lever in a piece of glass tubing containing 2.0—3.0 cc of Ringer's frog solution made with NaCl, 0.6 per cent; KCl, $CaCl_2$ and $NaHCO_3$, 0.02 per cent each; and glucose, 0.1 per cent. The glucose appeared to increase the endurance of the organ. Variation of only the KCl content down to 0.015 per cent was attempted, since potassium causes contraction of the rectus muscle (Chang and Gaddum 1933), but no essential difference was observed in the relaxation. The muscle contractions traced by the lever were usually of the magnitude 1 : 3.

After stretching of the muscle for 20—30 min. in ordinary Ringer's solution, the latter was replaced by a fresh solution containing physostigmine salicylate 1 : 100,000, which by inactivating the ChE of the muscle sensitises the muscle to ACh and facilitates the differentiation of ACh from other substances possibly present. Sensitisation was allowed to take place during 15 min., after which stabilisation of ACh was started with cautious doses. It was observed in preliminary experiments that the frog rectus was stabilised after three or four additions of ACh. The method is sensitive to 1: 2—10 million and is capable of demonstrating differ-

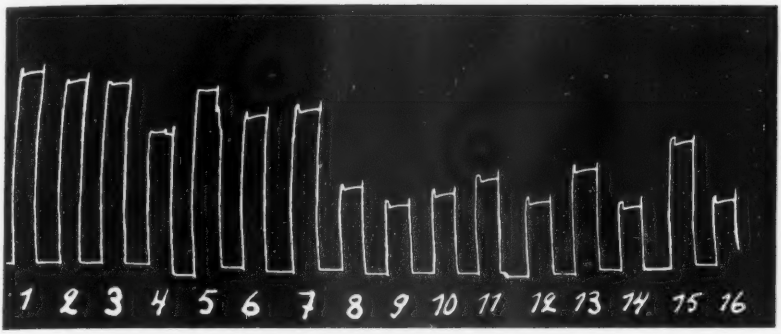


Fig. 1. — A typical graph for the reaction of frog rectus to ACh. 1, 3, 5, 7 and 15 = 0.5 μg ACh; 9 = 0.25 μg ACh; 10, 12, 14 and 16 = 0.3 μg ACh; 11 = 0.35 μg ACh; 13 = 0.4 μg ACh; 2, 4, 6 and 8 = no significance in this connection. Test object frog rectus.

ences in the amount of ACh down to 0.05—0.1 μg, as shown in Fig. 1. Regardless of the gradual, evenly progressing depression of the reaction (cf. contractions 1, 3, 5, 7 and 15 produced by 0.5 μg of ACh), the contractions remain comparable. Kahlson (1934 b) stated that differences down to 10 per cent in the amounts of ACh can be determined by this method. He has also drawn the »concentration-action curve», which is a nearly straight line in the area of optimum effect, making interpolation and quantitative estimation possible. In the present work, as in that of Kahlson, the comparable doses were found to be in ratios of about 1: 5, *i.e.*, amounts below 0.1 μg and over 0.5 μg of added ACh were no longer quantitatively comparable. The last two contractions in Fig. 2 show the deviations from the straight concentration curve due to such submaximal amounts. It is observed from the same figure that the volume of added ACh solution does not affect the amplitude of the contractions if the final concentration in the cuvette remains the same (No. 1—5).

In order to demonstrate different amounts of ACh, efforts were made to include controls made with known solutions as far as was allowed by the limitations of the technique and of the reactive capacity of the test object. The time required for one reaction series consisting of contraction, relaxation and rest is fairly long, being 15 (—30) min., and consequently the number of tests was unavoidably small. Possibilities for control, however, were greatly increased by carrying out parallel tests with muscle pairs, using the

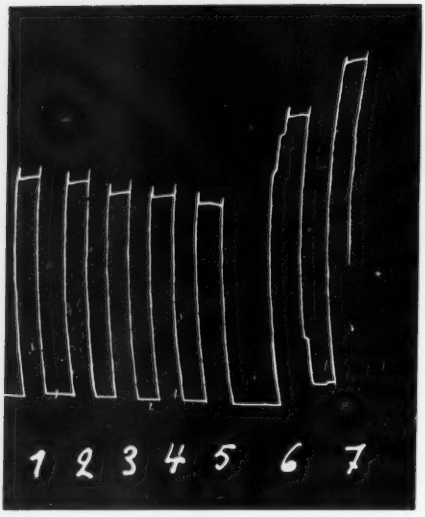


Fig. 2. — Deviations from the straight concentration curve due to submaximal ACh amounts. 1, 3 and 5 = 0.5 μg ACh (0.5 cc 1: 1,000,000); 2 = 0.5 μg ACh (0.3 cc 1: 600,000); 4 = 0.5 μg ACh (0.4 cc 1: 800,000); 6 = 1 μg ACh (1 cc 1: 1,000,000); 7 = 1.5 μg ACh (1.5 cc 1: 1,000,000). Test object frog rectus.

same timetable. As the reactions of a muscle pair are very similar and may frequently even be identical, several indirect controls are obtained in this way.

The procedure was as follows: Into the piece of glass tubing containing the sensitive muscle, usually 0.5 (—0.7 —1.0) cc of the test substance was pipetted. It was allowed to act for exactly 3 min., after which the contraction was interrupted by rinsing the test substance with six changes of Ringer's frog solution at intervals of ½ min., the last rinsing solution again containing physostigmine. Thus far 6 min. had passed, and since relaxation of the rectus usually required 12 min., the time of physostigmine action was 9 min. The muscle was then ready for a new reaction. The frog rectus retained its reaction power for 5—6 (4—7) hrs., which permitted up to 25—29 contractions. Frequently a rectus muscle which had been stored overnight in the refrigerator reacted well, sometimes even better than its pair which had been immediately used. Atropine used in small doses does not inhibit the ACh action on the frog rectus, but nicotine and d-tubo-curarine counteract the action.

The advantages of this test object are, above all, its highly specific reaction to ACh, uniformity of the reaction, the relatively short time required for relaxation, and the long endurance of the muscle.

Use of the Leech Dorsal Muscle. — Determination of ACh by means of the leech dorsal muscle competes with the above method in superiority. Fühner (1918 a, b) was the first to describe the effect of ACh on the physostigminised leech back and the method was develloped further by Minz (1932 a, b), who also described the procedure for quantitative determination of ACh with this test object (1932 a).

The leech test also is insignificantly disturbed by other substances which are present in biological material and which readily tend to produce harmful effects. The specificity of the method also excludes outright many possibilities of error which otherwise would be hard to eliminate. Specificity to ACh is a marked advantage of the two test objects here mentioned over, for instance, the frog heart, which also is sensitive (1 : 100—2,000 million, Kahlson 1933) and which is suited for the estimation of choline, or over intestine (Chang and Gaddum 1933, Kahlson 1934 a, b). As in the case of frog rectus, atropine does not affect the reaction of the leech to ACh but a small dose of nicotine or d-tubo-curarine abolishes it.

The technique of the leech test is in principle similar to the frog rectus test. The leech, however, does not react as satisfactorily as the frog rectus (Chang and Gaddum 1933), for the relaxation, although true to type, may frequently follow a non-horisontal basic line. It nevertheless possesses a certain advantage over the frog rectus which is a recommendation for its use, *i.e.*, of all the known methods it probably is the most sensitive specific one, being able to demonstrate ACh in dilutions as high as 1 : 2,000 million.

The leech was prepared according to the method of Minz (1932 b). The Ringer's solution differed slightly from that used for the frog rectus, containing NaCl, 0.6 per cent; KCl, 0.0075 per cent; $CaCl_2$ and $NaHCO_3$, 0.1 per cent each (Vartiainen and Kostia 1937). Contrary to its effect on the frog rectus, potassium causes relaxation in the leech, but a large amount of KCl, especially when used with a small amount of ACh, may produce contraction after having first initiated relaxation (Gollwitzer-Meier and Krüger 1934, Vartiainen and Kostia). Glucose was also employed in a part of the tests but it

was not found to alter the manner of reaction or the fatigue of the muscle. In an attempt to find the most suitable medium for perfusion of the muscle, tests were also made with Locke's solution, consisting of the usual mammalian solution in 1 : 1.4 dilution. However, activity of the muscle appeared to be unchanged. — For sensitisation, physostigmine salicylate was added to Ringer's solution in ratio 1 : 100,000 and 1 : 200,000. These two

Fig. 3. — Reaction of leech muscle to various ACh amounts. 1 and 3 = 0.02 µg ACh; 4 = 0.01 µg ACh; 5 = 0.005 µg ACh; 6 = 0.03 µg ACh. 2 = no significance in this connection. Test object leech muscle.

concentrations were found to have an equal sensitising action; dilution 1 : 250,000 possibly already had a slightly lesser action. The oxygen requirement of the leech muscle is extremely small in comparison with other isolated organs (Fühner 1918 b) and storage in the refrigerator overnight did not often seem to impair the usefulness of the muscle as a test object.

The test substance was allowed to act on the muscle for 2 min. only, as otherwise the relaxation time would have been greatly

prolonged. The sensitiveness of the test made it possible to demonstrate without difficulty ACh amounts of 0.01—0.02—0.03 μg, as seen in Fig. 3. In this case indeed the muscle was unusually sensitive and very clearly reacted to differences of 0.005 μg in ACh amounts. The varying amounts of ACh shown in the curve indicate that erroneous interpretations cannot arise from possible simultaneously occurring, continued sensitisation or already started fatigue of the muscle.

Relaxation of the leech dorsal muscle in the author's experiments was slower than reported in the literature. The time interval between tests was usually 30 min. and in some cases 25—20 min.

Being poikilothermal, the frog and the leech are both suitable for work at ordinary room temperature, which facilitates performance of the experiments. Their procurement is also fairly easy and inexpensive.

Use of Intestine. — Rat and mouse intestine was very rarely used as a test object in the author's experiments. When used, the principles of the method described by Page and Schmidt (1930) and Kahlson (1934 a) were employed. The contraction caused by ACh in intestinal smooth muscle differs from the reactions described above in being of short duration (Tiffenau and Scheiner 1939). Physostigmine is not capable of sensitising the intestine to ACh (Kahane and Lévy 1937 c).

The Taking of Aqueous Humour Sample. — One of the greatest difficulties in the performance of the experiments was the small amount of aq.h. available. At least three samples were needed for each experiment, *viz.*, 1) a non-incubated control, 2) an incubated sample, and 3) a sample incubated for a longer time, for confirmation of the test with sample 2). Since about 0.25—0.30 or at the most 0.35 cc of aq.h. was obtained from one rabbit eye, each of the three samples was usually less than 0.1 cc in amount.

Immediately before puncture the rabbit's eyes were anaesthetised by instillation of either 0.75 per cent pantocaine or 4 per cent cocaine into both eyes. This was usually done three times at intervals of about 1 min. Retrobulbar anaesthesia, which is recommended by some workers, was not attempted since this painful procedure would have tended to make the animals restless during the puncture,

which requires tranquil conditions. General anaesthesia would probably have been no better adapted for this work, for there was no evidence that its effect on the normal state of aq.h. would have been smaller than that of local anaesthesia. It is known, namely, that certain anaesthetics have various effects on the ocular pressure (Couadau and Campan 1952), and at least aether and urethane have been found to influence the ChE content of the blood (Ahlmark and Kornerup 1939), although a lethal dose of pentobarbital sodium has no effect on ChE activity (Schütz 1943 a, b). A tabulation of the relative inhibitive action exerted on ChE by anaesthetics used in ocular instillation has been prepared by de Grósz (1950), as follows:

Procaine	1
Larocaine	12
Percaine	28
Pantocaine	234

Thus pantocaine is in his opinion unsuitable for use in connection with ChE determinations. His samples were drawn under diocaine anaesthesia, and comparative tests were carried out with dogs anaesthetised with pernocton. The latter was not found by him to inhibit ChE in aq.h. and a similar observation regarding ChE in blood was made by Ahlmark and Kornerup (1939). In the opinion of de Grósz, the inhibitive action of local anaesthesia on ChE could be prevented only by retrobulbar anaesthesia. However, even the latter has probably some relationship with the ChE activity of aq.h., for also retrobulbar injection has been reported to influence the formation of ACh—ChE (de Roetth 1951, Arató and Arató 1952).

For performance of the puncture the rabbit was placed in a bag closed with a slide fastener and was held down on its side. The eye was fixated with ordinary conjunctival forceps only, as this seemed less liable to produce trauma than the regular fixation forceps. The cornea was penetrated obliquely at the limbus with a 19—20 gauge injection needle and aq.h. was aspirated into a tuberculin syringe, taking care not to injure the surrounding tissues, especially the iris. No blood entered the a.ch. after a well performed puncture.

Preparation of Solutions. — To ensure serviceability of the solutions used, $NaHCO_3$ and glucose were added to Ringer's solution just prior to commencement of each experiment and Tyrode's solution used in the intestine method was stored in the usual manner as two stock solutions which were combined when each experiment was started. ACh and other choline esters were stored in a 5 per cent primary sodium phosphate solution in the refrigerator, distributed into 1 mg tubes. A fresh batch of physostigmine stock solution was made every few days.

Controlling the Method. — In view of the numerous phases involved in the experiments there is reason to suspect the existence of several potential error factors. The following questions arise in this connection.

1) *Does Aqueous Humour Alone Produce a Contraction of the Test Object?* — In other words, does the aq.h. normally contain such amounts of ACh as are demonstrable by these methods? Engelhart (1931) has shown that 0.1—0.2 cc of rabbit aq.h. withdrawn under urethane anaesthesia will only produce in the frog heart a weak reaction which is not ascribable to ACh. Negative for ACh were also the results of Luco and Lissak (1938). On leech muscle Velhagen (1936) could demonstrate no ACh in the aq.h. of a physostigmine-treated, light-stimulated eye. In the experiments of Mazzella and Minz (1951), 1 cc of aq.h. from the eserinised and daylight-stimulated rabbit eye gave in the leech test a reaction corresponding to, at the most, 0.01 μg of ACh, and aq.h. from the eye of a rabbit held in the dark produced no reaction.

The author carried out control tests of the action of aq.h. on the frog rectus and the leech. Amounts up to 0.3 cc of primary aq.h. of the rabbit produced no reaction in the frog rectus. A similar negative result was obtained in the leech with 0.5 cc of rabbit primary aq.h. and with 0.4 cc of aq.h. from the fifth puncture (Fig. 4). These correspond to a weaker reaction than that of 0.01 μg of ACh, as contractions 3 and 5 in Fig. 4, which refer to these amounts of aq.h., show continued relaxation after the addition of aq.h. This was not the case after application of 0.01 μg of ACh, which, although it did not actually produce contraction, nevertheless prevented continuation of the relaxation. The curve simultaneously, shows, it is true, considerable fatigue of the muscle, but the quantitative differences are nevertheless clearly discernible.

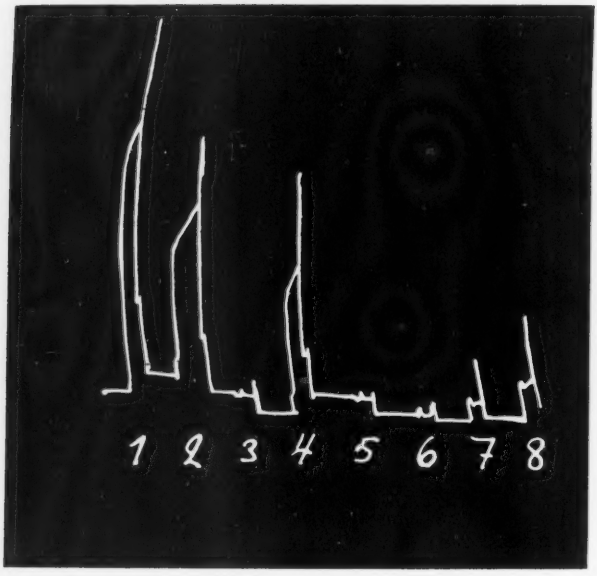


Fig. 4. — Effect of aq.h. on the leech muscle. 1 = 0.05 μg ACh; 2, 4 and 8 = 0.03 μg ACh; 3 = 0.5 cc rabbit aq.h.; 5 = 0.4 cc aq.h. of another rabbit; 6 = 0.01 μg ACh; 7 = 0.02 μg ACh. Test object leech muscle.

Since the tested amounts of aq.h. were several times larger than those usually employed in the experiments, it is obvious that aq.h. cannot lead to erroneous conclusions regarding muscle contractions.

2) *Will Physostigmine Produce Contraction of the Test Object?* — Physostigmine was used for inactivation of the ChE in the test object as well as of the ChE possibly present in the control sample. The final physostigmine concentration in the glass tubing was thus of the magnitude 1 : 20,000—30,000. It is a known fact that physostigmine also exerts a direct action on muscle. According to the studies of Führer (1918 c), a concentration of 1 : 200—400 was required before a strong contraction was obtained in the leech in 2—3 min. A dilution of 1 : 1,000 also caused a strong contraction, but the time required for the effect was 10—20 min. A slow but regularly contracting action was still exerted by physostigmine sulphate in dilution 1 : 2,000; dilution 1 : 5,000 contracted some of the leech muscles, and 1 : 10,000 only the most sensitive ones. The author's control tests confirmed these results also on frog rectus

which was not contracted in 3 min. even by a physostigmine concentration of 1 : 1,000.

These concentrations are so far removed from those used in the tests as to exclude any possibilities of error due to the contracting power of physostigmine.

3) *Spontaneous Hydrolysis of Acetylcholine.* — When it is a question of readily hydrolysed ACh and related substances, the possibility of spontaneous hydrolysis must be taken into consideration. Such hydrolysis may be a result of several factors, among which the hydrogen ion concentration should first be mentioned. For this reason ACh, MCh and BCh were stored in primary sodium phosphate solution, as was stated above.

In biological tests the enzyme concentration is usually so high that spontaneous hydrolysis cannot take place to any marked degree and tests for its determination are therefore unnecessary (Abdon and Uvnäs 1937). However, it seemed probable that this would not be the case in the present work. As it is known that the temperature coefficient of spontaneous hydrolysis is essentially greater than that of the enzymatic hydrolysis (Abdon and Uvnäs) and as the ACh had to be kept in the thermostat at +37° C for several hours, a comparison was made between the reactions given by so treated standard ACh solutions in Ringer and the original standard solutions. The longest period in the thermostat was 8 hrs., after which the solution probably produced a very slightly weaker contraction, but up to thermostat period of 4 hrs. the ACh solution was not found to cause a smaller contraction in the frog rectus than was obtained with the control. Spontaneous hydrolysis, therefore, appears to be so insignificant as to be outside the range of sensitivity of this method during the test periods used in the experiments.

Inhibition of the hydrolysis of ACh by physostigmine (0.1 cc of solution 1 : 1,000 in 0.5 cc of test fluid) also is evidence that contradicts the theory of spontaneous hydrolysis and proves that this is a case of hydrolysis by the esterase (contractions 11—20 in Fig. 5, page 34).

4) *Blood Content of the Aqueous Humour.* — As the samples of aq.h. were drawn by puncture and aspiration, it did not seem improbable that they could contain macroscopically occult blood which would be an erroneous source of ChE. It was, of course,

impossible to systematically examine each specimen, for every drop of aq.h. was needed for the test. Such a control was therefore made now and then, when permitted by the amount of puncture fluid or as a separate control. Since a microscopic examination using Bürker-Türk's counting chamber or Giemsa's staining revealed no blood cells in the rabbit and bovine aq.h., a chemical examination was also made by the simple procedure of Wagner. In tests with rabbit blood, concentrations as low as 1 : 50,000 gave a clearly discernible bluish--green colour on the object glass. When similarly tested, the rabbit and bovine aq.h. produced no visible colour reaction. On the other hand 0.5 cc of rabbit blood diluted 1 : 10,000 caused no hydrolysis of ACh even in 3 hours, although this amount was 10 times the amount of aq.h. usually employed in this work. A concentration of 1 : 1,000 was required before definite hydrolysis of ACh was obtained with the same volume in 3 hrs. (indefinite in 1 hr.). The results of these tests taken together exclude the possibility that blood in the aq.h. would contribute to the hydrolysis of ACh.

5) *Significance of pH in the Acetylcholine—Cholinesterase Reaction.* — The pH is a factor of great significance in ACh—ChE reactions. Especially a shift to the acid side decreases the activity of ChE. However, the pH is not as essentially significant in biological tests as in chemical reactions. When we take into consideration that the test conditions and the amounts of fluid used are nearly identical and the solutions correspond to biological conditions (Ringer's solution, ACh diluted with Ringer's solution, and aq.h.) the possible differences in the pH of the various test fluids cannot be very great. The pH values of human and rabbit aq.h. range between 7.15 and 7.77, and those of re-formed aq.h. are either the same or 0.1—0.3 more to the acid side (Süllmann 1951). Determinations of the pH would have caused a great deficit in the scanty amounts of test fluid available, and this procedure was therefore dispensed with as being of minor significance in this case.

CHOLINESTERASE ACTIVITY IN AQUEOUS HUMOUR

A. PRESENCE AND AMOUNT OF CHOLINESTERASE IN AQUEOUS HUMOUR

In Primary Aqueous Humour of the Rabbit. — Most of the experiments in this work were carried out with rabbit aq.h. To illustrate the course of the experiments a typical kymograph curve obtained with frog rectus is shown in Fig. 5.

For practical reasons the contractions are registered in the form of columns, for the curve obtained when the kymograph was running during the contraction was rather gradual and lengthy. The muscle was therefore allowed to contract while the kymograph was standing, and when rinsing was started the apparatus was put in operation for 15 sec. The use of columns facilitates comparison of the amplitude of the contractions, and depiction of the test results requires less space.

It will be observed from Fig. 5 that in the first four tests for ACh the muscle had become almost fully stabilised, for the difference between columns 3 and 4 is very slight. Observations made in these tests in general indicated that the difference between the fourth contraction and the following one would not have been any greater than the former, for contractions 4 and 5 were frequently of exactly the same magnitude.

The test substance was the primary aq.h. from a rabbit eye; the control was taken from the other eye, with physostigmine added before incubation. Contractions 5, 9, 11 and 15 produced by control substances containing physostigmine were slightly smaller than those given by the ACh control. The actual test for ChE activity is shown in contractions 11—18, in which 11 and 15 prove that no demonstrable hydrolysis of ACh had been produced during 1 hour's incubation by the aq.h. samples to which physostigmine had been added. Contractions 13 and 17, again, demonstrate that the aq.h. had definitely hydrolysed ACh during an incubation period of the same length.

This test thus demonstrates that the rabbit aq.h. hydrolyses ACh and that the hydrolysis is due to ChE, since it is inhibited by physostigmine.

The graph also includes controls made with ACh which had been stored in the thermostat for 1½ and 4¼ hrs., respectively (7 and 19). Hydrolysis was not clearly seen in these samples; in any case, the hydrolysis due to the esterase was of an entirely different magnitude. A very slight fatigue of the muscle could be observed at the same time in the form of gradually diminishing contractions. However, the muscle had performed faultlessly for 4 hrs.

A corresponding typical graph for tests with leech back is shown in Fig. 32 a, page 104.

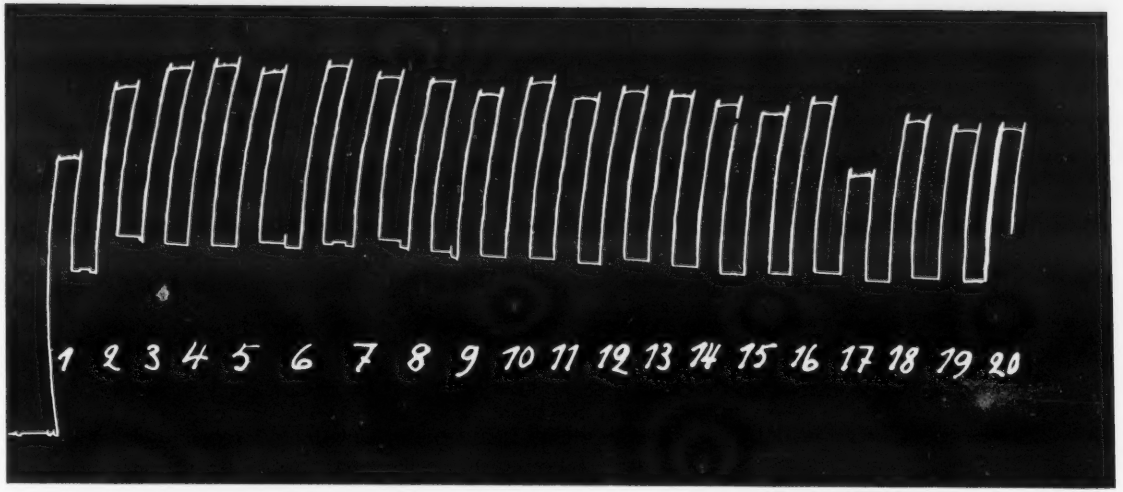


Fig. 5. — A typical graph in tests for ChE activity. 1—4, 6, 8, 10, 12, 14, 16, 18 and 20 = 0.5 μg ACh; 5, 9, 11 and 15 = 0.1 cc aq.h. + 0.1 cc physostigmine salicylate 1: 1,000 + 0.5 μg ACh (0.3 cc 1: 600,000), 5 and 9 same rabbit's aq.h., 11 and 15 another rabbit's aq.h.; 7=0.5 μg ACh after 1 ½ hrs. in thermostat at + 37° C; 13=0.1 cc rabbit primary aq.h. + 0.5 μg ACh; 17 = 13 incubated for 1 hr.; 19 = 0.5 μg ACh after 1 hrs. 15 min. in thermostat. Test object frog rectus.

It is characteristic of this muscle that it requires a considerably longer time to become stabilised than the frog rectus muscle, for it frequently continues to contract even after being stretched. The relaxation phase may last from ½ to 2½ hrs., but the time lost in waiting appears to be compensated by added sensitivity of the muscle. The amplitude of the contractions increases rapidly and sensitisation continues for a considerably longer time than in the case of the frog rectus. One cannot wait for stabilisation to set in.

In view of the continued sensitisation of the muscle, contraction 5 already creates a suspicion of some small degree of hydrolysis of the ACh. Estimated on the basis of the controls in the latter part of the graph, about 0.02 μg of ACh was hydrolysed in 1½ hrs. The latter portion of the curve, starting with No. 9, belongs to another test, but it shows clearly the effect of different doses of ACh on the basic line. No. 16 corresponds to a sensitivity to ACh concentration 1 : 250 million; the muscle had then been active for 7¼ hrs.

The peak on the columns indicates that contractions still continue after rinsing, for the action of ACh is cut off while the contraction is proceeding at a good speed. However, ACh does not disappear from the muscle instantaneously; the contraction therefore continues and the height of the peak thus produced is to some degree directly proportionate to the dosage employed.

Owing to the use of the biological method, the amounts of ACh and aq.h. and the other factors concerned are not systematically uniform. However, when several results are compared, many indirect conclusions may be drawn from them. Of the 56 tests listed in Table 1, 47 are definitely positive for ChE, 2 are doubtful and 7 negative. Tests 8, 9 and 10 raise in a way the number of positive results, for the determination in these tests was made from both eyes separately and both determinations were positive.

On the basis of these results it is evident that the rabbit aq.h. in most cases contains ChE. Quantitatively the amount is greatly variable, the lowest values being very minute. Indeed, the negative results should apparently be regarded as negative only because they probably are below the lowest limit of sensitivity of the technique.

The amount of aq.h. obtained by puncture varied between 0.19 and 0.35 cc, the a.ch. being frequently emptied completely. The body weight of the rabbit and the amount of aq.h. may have some directly proportionate correlation, for a large rabbit sometimes seems to have slightly larger eyes. However the amount of aq.h. obtained by puncture is frequently governed by chance,

TABLE 1

CHOLINESTERASE ACTIVITY IN PRIMARY AQUEOUS HUMOUR OF THE RABBIT

Test No.	Body Wt., kg	Aq. h. per Puncture, cc		Aq. h. per Test, cc		ACh per Test, μg	Hydrolysis of ACh, μg														ChE	Remarks
		R.E.	L.E.	R.E.	L.E.		30 min.	45 min.	1 h.	1 h. 15 min.	1 h. 30 min.	1 h. 45 min.	2 h.	2 h. 15 min.	2 h. 30 min.	2 h. 45 min.	3 h.	3 h. 15 min.	3 h. 30 min.	3 h. 45 min.	4 h.		
1	2.2		0.2		0.05	0.25		0.1														
2	2.1	0.5		0.05		0.5															+	
3	1.8			0.05		0.35															+	
4	2.3	0.47		0.15		0.5															+	
5	2.7	0.35	0.33	0.1		0.5						0.3					0.5				+	
6	2.1	0.35	0.25	0.1		0.5			0.15												+	
7	2.9	0.27		0.05		0.5			0.35											0.5	+	
			0.28		0.1	0.5			0.5												+	
8	2.1		0.31		0.1	0.5	0.1				0.3										+	
				0.1		0.5			0.2												+	
9	2.0	0.25	0.3	0.05		0.5	0.05		0.1												+	
		0.3			0.1	0.5	0.1		0.2												+	
10	2.4		0.3		0.05	0.5			0.1				0.2							0.3	+	
11	1.7		0.3		0.05	0.5			0.1				0.15								+	Phys.
12	2.0		0.25		0.05	0.5			0.15										0.3		+	Phys.
13	1.9		0.25		0.1	0.5			0.3												+	
14	2.5		0.3		0.05	0.5								0.2							+	
15	2.1		0.27		0.05	0.5	0.1				0.2		0.25								+	
16	1.9		0.25		0.05	0.5	0.05		0.1												+	Quant.
17	2.3		0.3		0.05	0.5			0.1												+	Phys.
18	2.1	0.27	0.28	0.1		0.5			0.2												+	Phys.
19	3.8	0.33		0.1		0.5			0.1				0.2								+	Quant.
20	2.2		0.26		0.1	1.0					0.6										+	
21	2.0		0.26		0.1	1.0						0.5									+	
22	2.1	0.23		0.1		0.5			0.2												+	
23		0.3		0.075		0.05															+	Phys.
24	3.3	0.3		0.075		0.05					0.02						0.025				+	Phys.
25		0.25		0.05		0.05					0.15										+	Wa—. Quant.
26		0.25		0.07		0.05			0.025												+	Wa—. Quant.
27	2.9	0.25		0.06		0.05			0.025												+	Quant.
28	2.3	0.25		0.05		0.02		?			?		?								+ ?	Quant.
29	2.1	0.2		0.05		0.02									0.01						+	Quant.

25		0.25		0.05		0.05	+		0.15						+	Wa—. Quant.
26		0.25		0.07		0.05		0.025								Wa—. Quant.
27	2.9	0.25		0.06		0.05		0.025							+	Quant.
28	2.3	0.25		0.05		0.02	?		?	+?					+?	Quant.
29	2.1	0.2		0.05		0.02					0.01				+	Quant.
30					0.05	0.05			0.02						+	
31					0.05	0.05				—					—	
32					0.05	0.05								0.01	+	Quant.
33					0.05	0.05		0.02			0.03				+	Quant.
34					0.05	0.05			0.015						+	Quant.
35					0.05	0.05			0.02						+	Quant.
36			0.28		0.05	0.05			—						—	Quant.
37			0.31		0.05	0.05	—?		+?						+?	Quant.
38			0.32		0.07	0.07	—		—						—	Quant.
39			0.3		0.05	0.05	+?		+						+	Quant.
40			0.21		0.05	0.05	+		0.01						+	Quant.
41			0.20		0.05	0.05		—		—					—	
42			0.22		0.05	0.05		—		—			—		—	Quant.
43			0.28		0.05	0.05	+								+	Quant.
44			0.27		0.05	0.05	+			0.01			0.03		+	Quant.
45			0.2		0.05	0.05		0.02	0.04						+	Quant.
46			0.25		0.05	0.05	+		0.01						+	Quant.
47	2.0		0.22		0.05	0.05		?				0.02			+	Quant.
48	2.2		0.23		0.05	0.05	+			0.03					+	Quant.
49	2.3		0.22		0.05	0.02	+		<0.01						+	
50	2.5		0.25		0.05	0.02	+			<0.005					+	Quant.
51	2.4		0.21		0.05	0.02	+		0.005						+	Quant.
52	2.6		0.26		0.05	0.02	+		0.005						+	Quant.
53	2.3		0.22		0.05	0.05	+		0.03						+	Quant.
54	2.2		0.23		0.05	0.05	—?		—?						—?	
55	2.5		0.19		0.05	0.05	+		0.02						+	
56	2.4		0.31		0.05	0.05	+		0.01						+	

Tests No. 31—53 made with »primary» aq. h. after instillation of propylene glycol into the eye. AntiChE had been instilled into the other eye for experiments in Part II.

Tests No. 54—56 made with »primary» aq. h. after instillation of arachis oil into the eye. AntiChE had been instilled into the other eye for experiments in Part II.

Phys. = Test included control with aq. h. + physostigmine.

Quant. = Amount of hydrolysed ACh was estimated from quantitative control test. In other tests the amount was interpolated on the basis of amplitude of the contractions.

Wa— = Test for blood in aq. h. with Wagner reagent was negative.

because of restiveness of the animal or for other technical reasons. It will be noted from the table that even such large amounts as 0.28, 0.32 and 0.35 cc may give negative results, while definitely positive results may be obtained with the smallest amounts. It therefore seems probable that the amount of aq.h. and the body weight have in any case no decisive bearing on the ChE activity of aq.h.

The sex of the animal also seems to have no significance, for positive results are given by both males and females. Most of the animals were males. This point is mentioned because attention has been drawn in the literature to differences in the ChE activity not only between different species of animals but also between the sexes, for instance in the case of rats (Burgen 1949 a). No conclusions could be drawn regarding the distribution of negative results, for the sex of the animals was not systematically noted down in the tests, and, furthermore, negative results were quite rare.

Estimation of the possible effect of anaesthetics on the ChE activity of aq.h. is considerably more difficult. Should there be reason to assume that the anaesthetics employed (cocaine and pantocaine) are, already after a few minutes' action, capable of producing an essential alteration in the quality of aq.h., there would first come to mind the inhibitive action of pantocaine on ChE and, consequently, the manifestation of this action as a reduction in the ChE activity. This possibility cannot be entirely excluded. For instance, pantocaine was regularly used starting with test No. 28, and, as is seen from the table, most of the ChE-negative results are to be found among these tests.

The varied effects of anaesthetics will be dealt with in greater detail in the Discussion (page 60).

The presence of ChE in the rabbit aq.h. was thus definitely demonstrated in these tests, but it remains to be solved in what amounts it is present. A ChE unit was defined by de Roetth (1950) as the amount of ChE in one millilitre of fluid which is capable of hydrolysing one milligram of ACh in one hour at + 37° C. It is unnecessary to calculate the amount of ChE in each test listed in Table 1, since many of the values are approximate only. For determination of the order of magnitude that is involved, it is sufficient to know that the tests with the frog rectus were usually made with 0.05 cc of aq.h. and 0.5 μg of ACh. As *c.* 0.1—0.2 μg

of ACh was hydrolysed in 1 hr., we obtain according to the above definition 0.002—0.004 ChE units as the amount present in rabbit aq.h. Amounts of 0 and 0.007 ChE unit are obtained at the extreme limits. Thus the amounts concerned are extremely minute indeed when compared with 20—200 units contained in the nervous and muscular tissue of the eye (de Roetth 1950) and for instance with the ChE content of the blood, in which the amounts reported vary according to the unit used. For instance in whole blood it is 13 units, of which 2 units are non-specific ChE (Gilman, Carlson and Goodman 1939).

Similar calculations made from the tests with the leech back give amounts of only 0.0002—0.0006 ChE unit. Although the two results show very small ChE amounts, their divergence at first seems unexpected, because the same aq.h. is in question. The amount of ACh hydrolysed was approximately the same, not in absolute quantity but as a percentage of the substrate. This observation, it is true, is compatible with the finding made already by Scheiner and Gautrelet (1939) that the amount of ACh has no influence on the rate of hydrolysis, since the ChE concentration alone affects the latter; in other words, the ChE—substrate ratio is the decisive factor, as has frequently been stressed in later investigations. Against this background it appears impossible to make a direct comparison of the ChE values obtained with the different techniques, as also is the case with the results of chemical and biological methods (Mahal 1938).

In Primary Aqueous Humour of Man. — Since ChE was found to be present in rabbit aq.h. as commonly as shown above, similar tests were carried out with human aq.h. for the purpose of comparison. In contrast to the negative findings of Matteucci (1947), de Grósz and Gorezky (1948) and Arató and Arató (1952), only de Grósz (1950) found it in the non-glaucomatous human eye. It is not the purpose in the present investigation to compare the normal and the glaucomatous eye in this respect but merely to obtain an answer to the question of whether or not the aq.h. of the human eye contains ChE.

The eyes of patients hospitalised for enucleation were selected at random for this study. The diagnosis and other pertinent facts in each case are shown in Table 2, in which these tests are tabulated.

TABLE 2

CHOLINESTERASE ACTIVITY IN PRIMARY AQUEOUS HUMOUR OF

Case No.	Diagnosis	Anaesthetic	Aq.h. per Punct., cc	Aq.h. per Test, cc	ACh per Test, µg	Hydrolysis of ACh, µg					ChE	Remarks
						30 m.	1 h.	1 h. 15 m.	1 h. 30 m.	2 h.		
1	Melanosarcoma oculi	Novocaine	0.2	0.05	0.5		0.05			0.1	+	Quant.
2	Tumor n. optici	Aether	0.25	0.1	0.5		0.05				+	Quant.
3	Melanosarcoma conjuntivae	»	0.19	0.05	0.5		0.1			0.2	+	Quant. Puncture 6 hrs. after enucl.
4	Glaucoma abs.	Novocaine	0.11	0.05	0.5		0.2				+	Quant.
5	Ablatio retinae	»	0.25	0.05	0.02	+		0.01			+	
6	Uveitis chr. post-traum.	»	0.2	0.05	0.02	0.005		0.015			+	Quant.
7	Uveitis post-traumatica	»	0.8	0.1	0.02	0.005			0.015		+	Quant.

The determinations were carried out with either frog rectus or leech back.

As is seen from the table, the results were positive in all cases irrespective of the anaesthesia used and the amount of fluid aspirated. Quantitatively too the ChE activity was of the same magnitude as that of rabbit aq.h. Fig. 6 shows the graph obtained in cases 1 and 2, including the quantitative ACh controls (14—17). Fig. 7 shows the graph for case 3. In this case a definite difference is seen between the incubated aq.h. sample on the one hand and the ordinary and thermostat ACh controls on the other hand.

Case 7 has been included in the table for the specific reason that the conditions in this case differed greatly from the usual. A very large amount of »aq.h.» was obtained by aspiration, but the puncture operation was interrupted after 0.8 cc had been withdrawn. Such a large amount of fluid obviously contained vitreous humour as a result of liquefied vitreous in the inflamed eye. Fig. 8 shows the curves obtained in the test made with this fluid.

It is rather unexpected that the hydrolysis of ACh in this case did not differ greatly from that in the other tests, for as late as

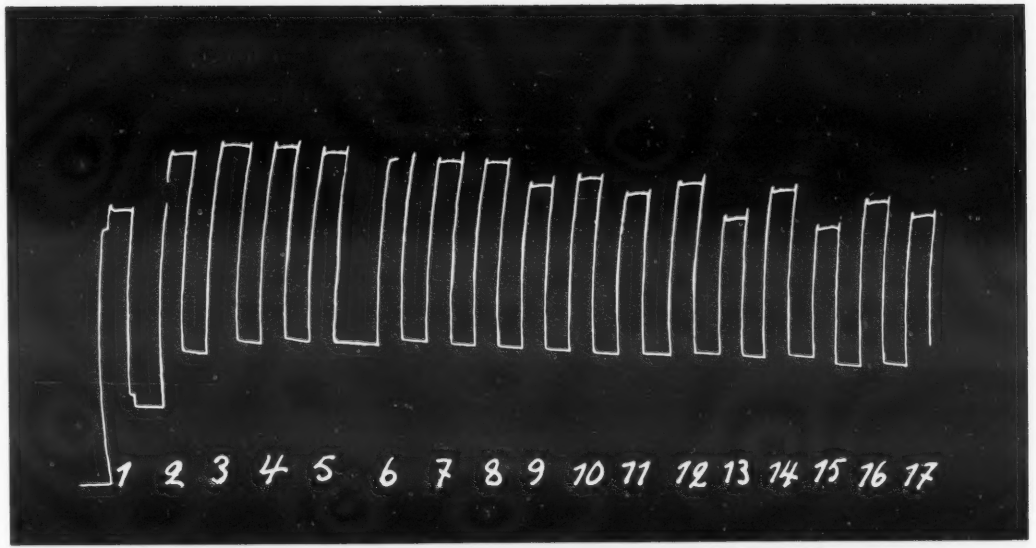


Fig. 6. — ChE activity in human aq.h. 1—4, 6, 8, 10, 12, 14 and 16 = 0.5 μg ACh; 5 = 0.05 cc aq.h. (case 1) + 0.5 μg ACh; 9 = 5 incubated 1 hr.; 13 = 5 incubated 2 hrs.; 7 = 0.01 cc aq.h. (case 2) + 0.5 μg ACh; 11 = 7 incubated 1 hr.; 15 = 0.4 μg ACh; 17 = 0.45 μg ACh. Test object frog rectus.

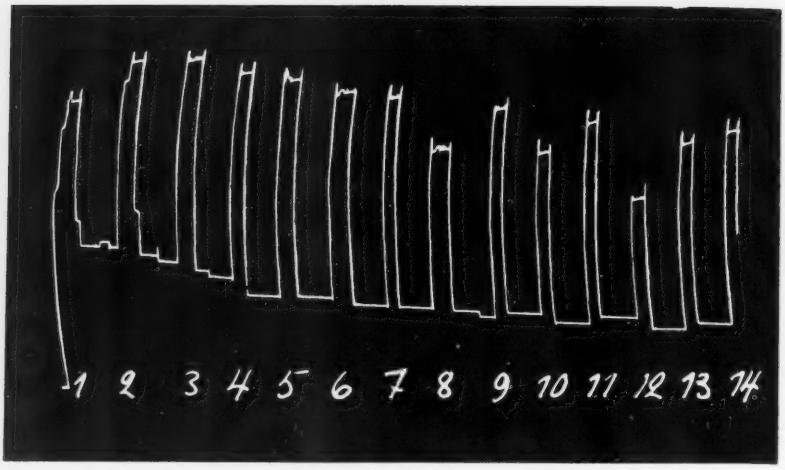
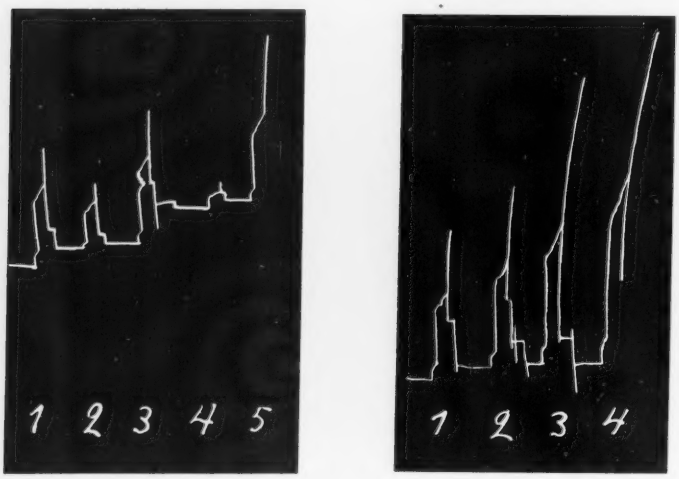


Fig. 7. — ChE activity in human aq.h. 1—3, 5, 7, 9, 11 and 14 = 0.5 μg ACh (0.5 cc 1: 1,000,000); 4 = 0.05 cc aq.h. (case 3) + 0.5 μg ACh (0.45 cc 1: 900,000); 8 = 4 incubated 1 hr.; 12 = 4 incubated 2 hrs.; 6 = 0.5 μg ACh (0.45 cc 1: 900,000); 13 = 6 after 2 hrs. 20 min. in thermostat; 10 = 0.4 μg ACh. Test object frog rectus.



a b

Fig. 8. — ChE activity in human aq.h. a) 1 = 0.1 cc aq.h. (case 7) + 0.02 μg ACh + 0.1 cc Ringer's solution; 2 = 1 incubated ½ hr.; 4 = 1 incubated 1 ½ hrs.; 3 and 5 = 0.02 μg ACh.

b) 1 = 0.1 cc aq.h. (case 7) + 0.02 μg ACh + .10 μg physostigmine salicylate (0.1 cc 1: 10,000); 2 = 1 incubated ½ hr.; 4 = 1 incubated 1 ½ hrs.; 3 = 0.02 μg ACh. Test object leech muscle.

1 ½ hrs. after incubation a slight contraction of the muscle was still seen [a) 4]. It seems probable that the suspicions expressed in the literature regarding the effect of vitreous body on the ChE content of aq.h. withdrawn by puncture need not be given too much significance, even if Weve and Fischer (1939), who found no ChE in aq.h., were able to demonstrate it in the vitreous humour. Furthermore, in normal puncture the presence of vitreous humour in the aq.h. is highly questionable. The control made of the hydrolysis-inhibiting action of physostigmine with a pair of muscles (Fig. 8 b) is again a proof of the hydrolysis of ACh by the esterase.

In Primary Aqueous Humour of the Cat. — As the values earlier reported in the literature for ChE activity in the aq.h. and elsewhere in the eye have been greatly variable from one species of animal to another, determinations were carried out by the above methods on certain animals for the purpose of comparison.

As a test animal, the cat is considerably more difficult than the rabbit to handle during puncture of the a.ch., if this is done without general anaesthesia. And since general anaesthesia possibly has a different effect on the ChE activity of aq.h. than local anaesthesia, samples were taken under both conditions. For a successful puncture without general anaesthesia, a very firm pressure on the cat's head is required to keep the animal quiet. However, whether the anaesthesia employed is local or general, there is the common difficulty that blood very readily enters the a.ch. during aspiration. Therefore it is not possible to use all of the aq.h. of the cat, which is rather abundant in comparison with rabbit and human aq.h. Rapid aspiration of only a part of the cat aq.h. yielded bloodless samples (*i.e.*, samples with a blood content at least below 1 : 50,000 = Wagner:—).

The details of the samples listed in Table 3 vary in almost all respects. With a few exceptions they are definite evidence of ChE activity in the cat aq.h. It was not possible in all the tests to calculate the number of ChE units present, but calculations made in a few cases gave values that did not exceed those of the rabbit and hardly amounted to one thousandth part of one unit. The type of anaesthesia used appeared to have no effect — at least no decisive effect — on the positivity of the test for ChE, and there appeared to be no reason to make quantitative comparisons in view of the

TABLE 3

CHOLINESTERASE ACTIVITY IN PRIMARY AQUEOUS HUMOUR OF THE CAT

Test No.	Aq.h. per Puncture, cc	Blood in aq.h.	Aq.h. per Test, cc	Substrate			Hydrolysis of Substrate, μg									ChE	Test Object
				ACh, μg	BCh, μg	MCh, μg	30 min.	1 h.	1 h. 15 min.	1 h. 30 min.	2 h.	2 h. 15 min.	2 h. 30 min.	2 h. 45 min.	3 h. or more		
1	0.8—1.0		0.1	0.05				0.02					0.04			+	Leech
2	1.0	?	0.3		5								4			+	»
3	>0.6	Macr.—	0.2		5					0.2					4		»
4	0.9	E+	0.4		5		3									+	»
5	0.9	E+	0.4		5		1—2									+	»
6	1.6 [1]	E—	0.5		5			1—5								+	»
7	0.9 [1]	E—	0.15	0.5				0.1			0.2					+	Frog
8	0.65	E—	0.3	0.5				0.2								+	»
9	>0.75 [1]		0.25	0.5					0.1		0.2					+	»
10	1.20	Wa—	0.3		5					4		5				+	Leech
		Wa—	0.02	0.05										+?	0.1	+	»
11	0.55	Wa—	0.05	0.05						0.01					0.03	+	»
		Wa—	0.1								0.025					+	»
12	1.0 / 1.2 [2]	Macr.—	0.3	1				+?			+					+	Intestine
		»	0.35			2		—			—					—	»
13	0.47		0.1	0.04				+?					+			+	Leech
14	0.32	Wa—	0.1	0.04				+							+		»

[1] Total from both eyes.
[2] Total from three cats.
With the exception of tests No. 13 and 14, in which pantocaine was employed, the anaesthetic was aether or urethane.
E = erythrocytes, Macr. = macroscopic blood, Wa = Wagner.

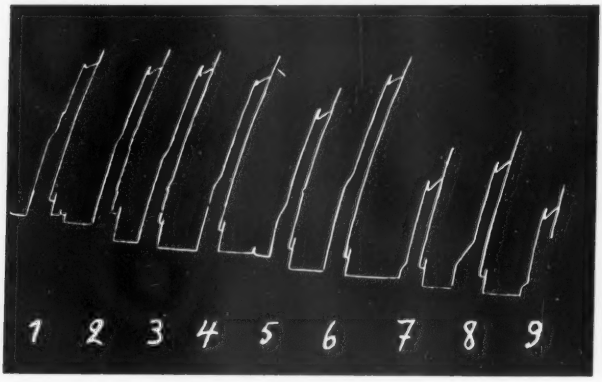


Fig. 9. — ChE activity in cat aq.h. 1, 4 and 6 = 0.05 µg ACh; 2 = 0.05 cc cat aq.h. (test 11) + 0.05 µg ACh; 5 = 2 incubated 1½ hrs.; 9 = 2 incubated 3½ hrs.; 3 = 0.1 cc same aq.h. + 0.05 µg ACh; 7 = 3 incubated 2 hrs.; 8 = 0.03 µg ACh. Test object leech muscle.

minimal amounts of ChE involved. The listing of several substrates in this table is due to the inclusion of tests for the typing of ChE, which at the same time were used to demonstrate ChE in primary aqueous humour. The aq.h. used in the last test was not wholly primary, but the test has been included in this table as 2½ months had elapsed since the previous puncture. A test on the ChE-inhibiting action of physostigmine was made in connection with tests 1, 2, 3, 7, 9, 13 and 14. In tests 4, 7, 8 and 12 the hydrolysed amount of ACh was confirmed by a quantitative control; in the other tests this amount was obtained by interpolation.

Fig. 9 is the graph for test 11, showing the curves for ACh hydrolysis by 0.05 and 0.1 cc of aq.h. It is seen that a twice as large amount of aq.h. hydrolyses an approximately twice as large amount of ACh.

In Primary Aqueous Humour of Some Slaughter Animals. — The samples for these tests were taken in connection with the slaughter of the animals, and were drawn either from the unconscious animal or immediately after death. Depending on the size of the eye, the amount of puncture fluid obtained was *ad* 2—2½ cc; the a.ch. was not completely emptied. Strictly scientifically considered, this aq.h. is no longer primary, for postmortal changes begin immediately after death. From the practical point, however, it is hardly of significance, for in many cases it has only been a question of

seconds, sometimes of a few minutes, and in rare cases of hours. On the other hand, it is difficult to estimate the possible changes due to the slaughter operation itself. Especially the electric shock given to the pig prior to slaughter may be a cause of suspicion in this respect. Sample-taking from the pig is technically most difficult also in other respects, for puncture of the a.ch. of a pig shaking under the shock does not fill the demands set upon faultless tech-

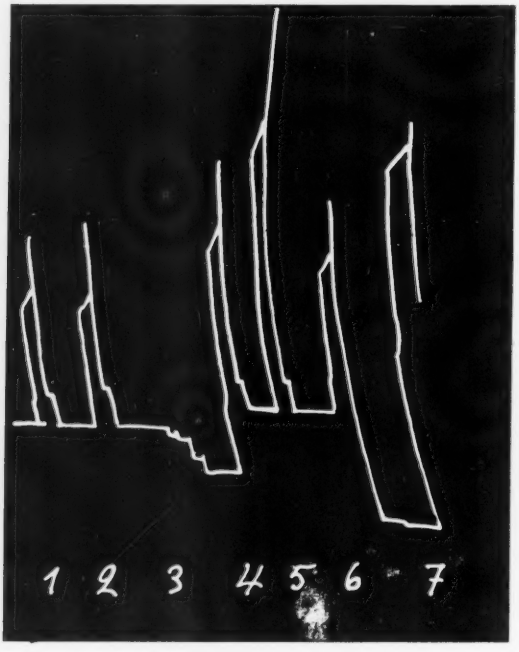


Fig. 10. — ChE activity in bovine aq.h. 1 = 1 cc bovine aq.h. + 0.1 μg ACh; 3 = 1 incubated 1 hr.; 2 = 0.1 μg ACh; 4 = 1 cc same aq.h. + 0.1 μg ACh + 0.1 cc physostigmine salicylate 1: 1,000; 7 = 4 incubated 2 hrs. 40 min.; 5 and 6 = no significance in this connection. Test object leech muscle.

nique. However, since the aq.h. used in these tests was not drawn secondarily following a primary puncture, the samples were considered to be primary aq.h.

The results of the tests are shown in Tables No. 4—7, each species of animal being dealt with in a separate table. A typical kymograph curve is also given for each group in Figs. 10—12.

Fig. 10 is the curve for test 7, in which 1 cc of *bovine aq.h.* hydrolysed in 1 hr. the entire amount of substrate used, *i.e.*, 0.1 μg

TABLE 4

CHOLINESTERASE ACTIVITY IN PRIMARY BOVINE AQUEOUS HUMOUR

Test No.	Time post mortem h.	Time post mortem min.	Aq.h. per Test, cc	ACh per Test, μg	Hydrolysis of ACh, μg: 30 min.	45 min.	1 h.	1 h. 15 min.	1 h. 30 min.	1 h. 45 min.	2 h.	2 h. 15 min.	ChE	Test Object	Remarks
1		5—10	0.1	0.5		?				0.1			+	Frog	Cow
2		»	0.1	0.5			+?				+		+	»	»
3		5	0.1	0.5			+				+		+	»	Ox [1]
4		30	0.1	0.5			+?				+?		+?	»	Cow
5	1		0.1	0.5			0.1					0.2	+	»	Ox
6		[2]	0.1	0.5							0.1		+	»	Cow
7	1		1.0	0.1			0.1 Compl.						+	Leech	8 Cows Wa—
8	2		0.2	0.05			0.05 Compl.						+	»	Cow
9		1 2	0.1	0.5			+?				+?		+?	Frog	Calf
10		5	0.1	0.5							0.5		+	»	»
11		5	0.1	0.5							0.25		+	»	»
12	2?		0.2	0.05			0.05 Compl.						+	Leech	»

[1] Slaughtered by shooting twice in the head.
[2] Unconscious.
Wa = Wagner.
Compl. = completely hydrolysed.

ACh (contractions 1 and 3). The following portion of the curve shows the control containing physostigmine; in 2 hrs. 40 min. no hydrolysis of ACh whatsoever was observed, not to speak of complete hydrolysis of ACh.

In Table 4, 10 positive tests out of 12 are evidence that ChE is commonly present in bovine aq.h. The two doubtful results were at the limit of sensitivity of the technique. Calculated on the basis of the data in the table, the maximum ChE activity was 0.0025 unit (test 10). Thus the ChE values in also these tests do not exceed thousandths of one unit.

All six tests in Table 5 demonstrate that ChE is present in *horse aq.h.* The tests were carried out with frog rectus, but estimation of the amount of ACh hydrolysis was difficult in cases 5 and 6, as the amount of substrate was greater than usual and no quanti-

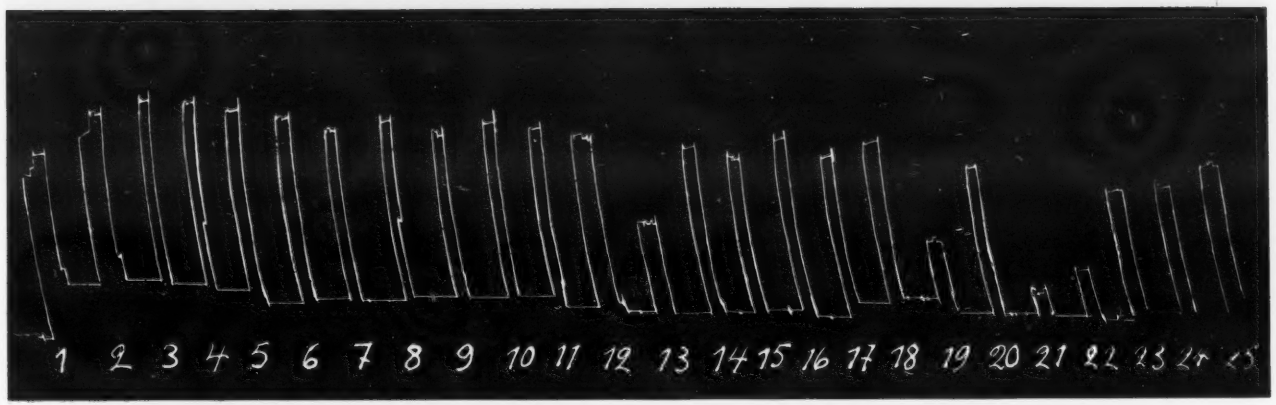


Fig. 11. — ChE activity in horse aq.h. 1—4, 6, 8, 10, 12, 14, 16, 18, 20 and 25 = 0.5 μg ACh; 5 = 0.1 cc horse aq.h. (test 4) + 0.5 μg ACh; 13 = 5 incubated 2 hrs.; 21 = 5 incubated 4 hrs.; 7 = 5 + 0.1 cc physostigmine salicylate 1: 1,000; 15 = 7 incubated 2 hrs.; 23 = 7 incubated 4 hrs.; 9 = 0.1 cc sheep aq.h. (test 5) + 0.5 μg ACh + 0.1 cc physostigmine salicylate 1: 1,000; 17 = 9 incubated 2 hrs.; 11 = 0.1 cc same sheep aq.h. + 0.5 μg ACh; 19 = 11 incubated 2 hrs.; 22 = 0.25 μg ACh; 24 = 0.4 μg ACh. Test object frog rectus.

TABLE 5

CHOLINESTERASE ACTIVITY IN PRIMARY AQUEOUS HUMOUR OF THE HORSE

Test No.	Time post mortem, h. min.	Aq.h. per Test, cc	ACh per Test, μg	Hydrolysis of ACh, μg				ChE	Test Object
				1 h.	2 h.	3 h.	4 h.		
1	30	0.1	0.5	0.1	0.2			+	Frog
2	30	0.1	0.5		0.15		0.25	+	»
3	30	0.2	0.5		0.2			+	»
4	1	0.1	0.5		0.2			+	»
5	during bleeding	0.2	1.0		+			+	»
6	(*c.*1 min.)	0.2	1.0		+			+	»

tative controls were made of ACh amounts less than 1 μg. In the other cases the ChE activity was of the same order of magnitude as in the previous series.

The kymograph curve for test 4 is seen in Fig. 11. The hydrolysis of ACh is obvious (contractions 5, 13 and 21). The control containing physostigmine also gave a column that is insignificantly — 0.1 μg at the most — below normal in height, for it had been very slightly lower than that of standard ACh throughout the entire test. ACh controls 22 and 24 are an aid in the estimation of the hydrolysed amount of ACh.

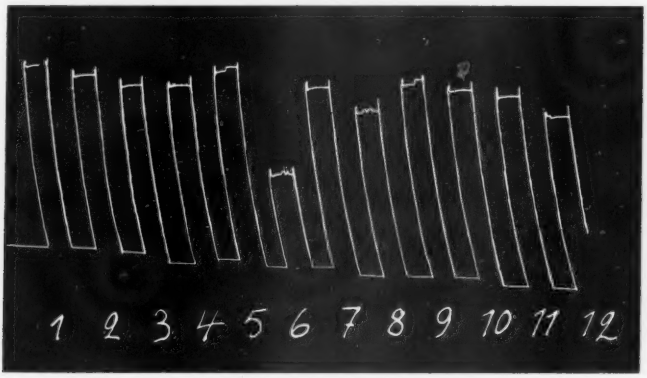


Fig. 12. — ChE activity in pig aq.h. 1, 3, 5, 7, 9 and 11 = 0.5 μg ACh; 2 = 0.1 cc pig aq.h. (test 1) + 0.5 μg ACh; 6 = 2 incubated 1 hr.; 4 = 2 + 0.1 cc physostigmine salicylate 1: 1,000; 8=4 incubated 1 hr.; 10=0.05 cc pig aq.h. (test 2) + 0.5 μg ACh; 12 = 10 incubated ½ hr. Test object frog rectus.

Fig. 12 shows the kymograph curve obtained in test 1 with *aq.h. of the pig*. As was the case in the recent test with horse aq.h. and in a number of other tests, there was some slight hydrolysis in the physostigmine control; however, this hydrolysis was of a magnitude quite different from that in the actual test.

TABLE 6

CHOLINESTERASE ACTIVITY IN PRIMARY AQUEOUS HUMOUR OF THE PIG [1]

Test No.	No. of Animals	Aq.h. Collected, cc	Aq.h. per Test, cc	ACh per Test, μg	Hydrolysis of ACh, μg							ChE	Test Object
					30 min.	45 min.	1 h.	1 h. 15 min.	1 h. 30 min.	1 h. 45 min.	2 h.		
1	1	0.25	0.1	0.5			0.2					+	Frog
2	1	0.2	0.05	0.5	+							+	»
3	3	0.6	0.1	0.5							0.3	+	»
4	1	0.35	0.1	0.5						0.3		+	»
5	1	0.5	0.1	0.5							0.3	+	»
6	2	0.8	0.1	0.5							>0.3	+	»

[1] All samples were drawn while the pig was under electric shock.

All the tests with pig aq.h. (Table 6) were positive for ChE, but no controls were made of possible blood in the aq.h. The presence of blood did not necessarily play a part in the hydrolysis of ACh, for it was of the same magnitude as in the preceding test series. The differences in the columns between the controls and the actual tests are very distinct; however, the amount of aq.h. per test was greater than, for instance, in the tests with rabbit aq.h.

TABLE 7

CHOLINESTERASE ACTIVITY IN PRIMARY AQUEOUS HUMOUR OF THE SHEEP

Test No.	Aq.h. per Puncture, cc	Aq.h. per Test, cc	ACh per Test, μg	Hydrolysis of ACh, μg			ChE	Test Object
				1 h.	1 h. 30 min.	2 h.		
1	0.5	0.1	0.5	0.1			+	Frog
2	1.0	0.1	0.5			0.3	+	»
3	0.9	0.1	0.5		0.15	0.2	+	»
4	0.6	0.1	0 5			0.15	+	»
5	0.8	0.1	0.5			0 2	+	»

Tests with *sheep aq.h.* are shown in Table 7. All samples were withdrawn while the animal was unconscious before slaughter, or within 1 min. after slaughter.

Test 5 with sheep aq.h. is included in Fig. 11 on page 48. Definite hydrolysis of ACh is seen in the test and the physostigmine control does not clearly show hydrolysis.

All of the 5 tests in Table 7 were positive for ChE, and the ChE activity was of the same magnitude as in the preceding test series.

Summary of Tests with Primary Aqueous Humour. — The results of the 7 test series described above were in agreement regarding both the presence and amount of ChE in primary aq.h. Thus ChE activity in the aq.h. was demonstrated in 47/56 tests with rabbit, 7/7 with human, 14/14 with cat, 10/14 with bovine, 6/6 with horse, 6/6 with pig and 5/5 tests with sheep aq.h. The remaining tests, which were doubtful or seemed definitely negative, were probably negative only because of the inadequate sensitivity of the technique. A considerable problem in an investigation of this kind, in which the results move at the extreme limits of methodic sensitivity, is the difficulty of demonstrating the smallest amounts concerned, as this requires the coinciding of a very sensitive and uniformly reacting test object on the one hand and successfully drawn test samples on the other hand.

In Secondary Aqueous Humour. — It has been demonstrated by several investigators that secondary, plasmoid aqueous humour contains ChE and that its amount is definitely greater than in primary aq.h. Various theories on its origin have also been presented (Brückner 1943 a, Jaffe 1948, de Roetth 1950 and Toda 1952). Regardless of this, an attempt was made in the present work to obtain a personal opinion of the ChE activity of secondary aq.h. for comparison with the results obtained with primary aq.h.

The test animals were rabbits and the results are shown in Table 8. With one exception, all the tests were positive for ChE. The negative result was given by a secondary aq.h. sample drawn 48 hrs. after the first puncture. It seems probable that the ChE activity regains to a great extent the normal level within this time, and if it normally was very low in this rabbit the negative test result is readily understood. Estimation of the amount of activity

TABLE 8

CHOLINESTERASE ACTIVITY IN SECONDARY AQUEOUS HUMOUR OF THE RABBIT

Test No.	Aq.h. per Puncture, cc		Aq.h. per Test, cc	ACh per Test, μg	Hydrolysis of ACh, μg						Interval between Punctures		ChE	Test Object
	R.E.	L.E.			30 min.	1 h.	1 h. 15 min.	1 h. 30 min.	2 h.	2 h. 15 min.	h.	min.		
1	0.33	0.25	0.1	0.5	—	—		—		—	48		—	Frog
2		0.3	0.05	0.5		+?			0.1		23		+	»
3		0.28	0.05	0.5				0.5			4	45	+	»
4		0.28	0.05	0.5		0.5					4		+	»
5		?	0.05	0.5		0.5					4	40	+	»
6		0.2	0.05	0.5	0.5						5		+	»
7		?	0.1	0.5			0.4?				24		+	Intesti
8	0.23		0.1	0.5		0.3					48		+	Frog
9		0.23	0.065	0.05	0.02			0.04			22		+	Leech

in the ChE-positive cases is difficult because of the few tests possible, but generally a definitely greater amount of ACh was hydrolysed than in the tests with primary aq.h. For instance in test 6 the total amount of ACh was hydrolysed already in 30 min., and it is very possible that this would also have been the case in tests 3, 4

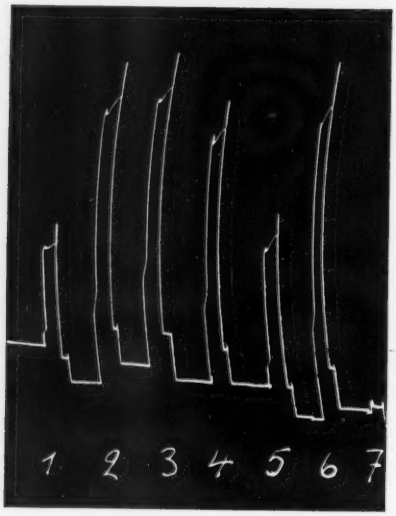


Fig. 13. — ChE activity in rabbit secondary aq.h. 1 = 0.03 μg ACh; 2, 3 and 6 = 0.05 μg ACh; 4 = 0.065 cc rabbit secondary aq.h. + 0.05 μg ACh; 5 = 4 incubated ½ hr.; 7 = 4 incubated 1½ hr. Test object leech muscle.

and 5. When the result of test 6 is converted to ChE units we obtain the value of 0.02 unit, which is approximately 10 times as high as that obtained for primary aq.h. As may be understood, even this need not be the maximum value obtainable, for these tests include none made with incubation times of less than 30 min. or with puncture intervals below 4 hrs., which evidently tend to enhance ChE activity.

Fig. 13 shows the hydrolysis of ACh in test 9.

TABLE 9

CHOLINESTERASE ACTIVITY IN TERTIARY AQUEOUS HUMOUR OF THE RABBIT

Test No.	Aq.h. per Puncture, cc		Aq.h. per Test, cc	ACh per Test, µg	Hydrolysis of ACh, µg				Interval between Punctures		ChE	Test Object
	R.E.	L.E.			30 min.	1 h.	1 h. 30 min.	2 h.	1—2 h. min.	2—3 h. min.		
1	0.3	0.15	0.1	0.5	?		+	0.2	48	16	+	Frog
2	0.28	0.3	0.1	0.5	—	—	—	—	96	72	—	»
3	0.35		0.1	0.5		+?		+	96	74		»
										16	+ [1]	
4	0.25		0.05	0.5		0.3		0.5	23 30	22 30	+	»
5		0.3	0.05	0.5		+		0.1	23	21 30	+	»

[1] Quaternary aqueous humour.

In Tertiary Aqueous Humour. — Although it was hardly presumable that tertiary aq.h. would greatly differ from the secondary, a few tests were carried out with this fluid (Table 9). However, the results obtained allow us to draw no definite conclusions but only some inferences of a general nature. The ChE activity did not exceed that of secondary aq.h., the opposite being rather the case. Increased ChE amounts are no longer demonstrable a few days after the second puncture (test 2), whereas ChE is again found after a shorter interval, as in test 3, which is a continuation of test 2 and was made with quaternary aq.h.

B. SPECIFICITY OF CHOLINESTERASE IN AQUEOUS HUMOUR

The type of ChE may be determined in the following two ways:

a) By employing different choline esters in known concentrations, since it is known that certain esters are most readily hydrolysed by their »own» esters.

b) By employing the same substrate in different concentrations, since it is known that each enzyme has a specific optimum concentration of substrate.

As the results of both methods are compatible (Nachmansohn and Rothenberg 1944, Augustinsson 1949, Augustinsson and Nachmansohn 1949 a and b), the use of either procedure alone is adequate. In view of the low ChE activity here present, the latter technique did not seem practicable. For this reason the choice fell upon the use of different esters, as employed by de Roetth (1950). He carried out his tests with propionylcholine and butyrylcholine, whereas acetyl-β-methylcholine (MCh) and benzoylcholine (BCh), which also have been commonly used (Augustinsson 1948), were selected for the present work.

Determination of Acetyl-β-Methylcholine and Benzoylcholine. — The nicotine-like action of MCh is very weak (Tiffenau and Scheiner 1939), and no response from the frog rectus muscle was obtained even by the writer with a dose of 100 μg, which in fact would have been too large for the present tests. The techniques used in the preceding tests were therefore not serviceable and it was necessary to attempt to carry out the tests with smooth-muscle preparations, *i.e.* intestine. The mouse intestine (Page and Schmidt's method, 1930) responded to ACh doses of 0.1—0.5 (—1.0) μg, corresponding to the sensitivity of intestine pieces to ACh concentration 1: 30 million, as mentioned in the literature, and to the sensitivity of frog rectus muscle reported. However, the amount of MCh required was 2—2.5 times the amount of ACh. As, furthermore, the capacity of the cuvette employed in this work was 5 cc, or larger than in the frog tests, the amount of substrate was relatively even greater than in the preceding tests. The rat intestine is frequently twice as sensitive as the mouse intestine but at the same time much more restless, spontaneous contractions being common. Furthermore, the large amount of aq.h. collected in order to obtain a sufficient amount of ChE and added, in a small vessel, to a sensitive mammalian organ requiring an even temperature, and other substances possibly present in the aq.h., caused so many irregularities in the contractions that the curves could not always be considered reliable. However, there is no doubt but that the intestine is a good test object (Bentley and Shaw 1952), when a better source of enzyme than aq.h. is used.

BCh, on the other hand, caused a weak contraction in the frog rectus, 100 μg producing a contraction that corresponds approximately to 0.5 μg of ACh and the ratio of effect thus being about 200: 1. The leech, which also is sensitised by physostigmine to BCh 6-fold (Kahane and Lévy 1937 a, d), was a somewhat better test object but the usual test dose of 5 μg of BCh was still excessive for the weak ChE activity in aq.h. Intestine, on the other hand, did not react to BCh even in concentration 1: 20,000, equal to 1,000 μg of BCh under these conditions.

Determinations of the specificity of ChE in human aq.h. were omitted in view of the difficulty of procuring this fluid, especially in the large amounts required at one time. An attempt was therefore made to elucidate the subject of ChE types by tests with aq.h. of the cow, rabbit and cat, using the leech as test object.

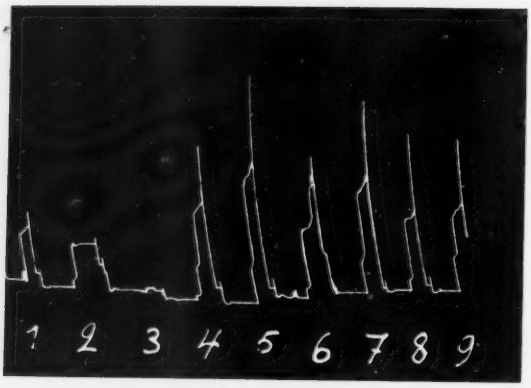
TABLE 10

EXPERIMENTS ON SPECIFICITY OF CHOLINESTERASE IN BOVINE AQUEOUS HUMOUR

Test No.	Time post mortem, h.	Aq.h. per Test, cc	Substrate		Hydrolysis of Substrate, μg								ChE	Non-Specific ChE
			ACh, μg	BCh, μg	1 h.	1 h. 15 min.	1 h. 30 min.	1 h. 45 min.	2 h.	2 h. 15 min.	2 h. 30 min.	2 h. 45 min.		
1[1]	1	1	0.1		0.1								+	
		2.5		10	—?									—
2	2—3	0.2	0.05		0.05								+	
		3		5							—?			—?
3	2—3	0.2	0.05		0.05								+	
		3.0		5							—			—
4	1	0.01	0.05			—				—			—	
		1.25		5		—				—				—
5	1	0.1	0.05						—			—	—	
		2.25		5					—					—
6		0.5		2	+?				+?					+?

[1] Random test for blood was Wagner: —.

Tests on the Specificity of Cholinesterase in Bovine Aqueous Humour. — Table 10 shows the results of the tests with bovine aq.h. but the conclusions that may be drawn are rather limited. The hydrolysis of ACh is included for comparison in order to give confirmation of the presence of ChE. Although none of the tests showed



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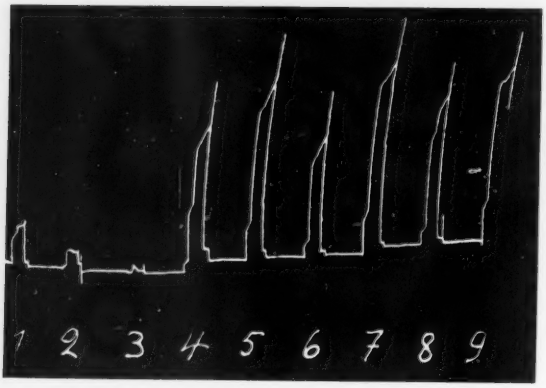


b

Fig. 14. — Possible BCh-hydrolysing effect of bovine aq.h. a) 1 = 3 cc bovine aq.h., time of action 1 min.; 2 = 1 cc bovine aq.h., 2 min.; 3 = 1 cc Ringer's solution, 2 min.; 4 = 2 μg BCh, 2½ min.; 5 = 0.5 cc bovine aq.h. + 2 μg BCh, 2 min.; 6 and 8 = 2 μg BCh; 7 = 5 incubated 1 hr.; 9 = 5 incubated 2 hrs. Time of action in 5—9, 2 min.

b) 1 = 2 cc bovine aq.h., 1½ min.; 2 = 0.5 cc bovine aq.h., 2 min.; 3 = 1 cc Ringer's solution, 2 min.; 4 = 2 μg BCh, 2½ min. [as a) 4]; 5 = 0.5 cc bovine aq.h. + 2 μg BCh + 0.1 cc physostigmine salicylate 1: 5,000; 6 and 8 = 2 μg BCh; 7 = 5 incubated 1 hr.; 9 = 5 incubated 2 hrs. Time of action in 5—9, 2 min. Test object leech muscle.

hydrolysis of BCh, these results can serve as relative evidence only, for, although 0.2 cc of aq.h. was capable of hydrolysing 0.05 μg of ACh in 1 hr., BCh was not only present in 100-fold amount but was also more difficult to hydrolyse than ACh [*e.g.*, the ratio of hydrolysis of ACh and BCh in leech tests was 1: 0.17 and in frog

tests 1: 0.35 (Kahane and Lévy 1937 d)]. Thus a 15-fold volume of aq.h. was not necessarily adequate to produce appreciable hydrolysis in 5 μg of BCh even during an incubation time that was 2¼ times as long. On the other hand, it was not possible to increase the aq.h. dose further, as the muscle already lay in almost pure aq.h. during the test and as this amount of aq.h. already was capable of producing a distinct contraction. This is shown by controls 1 and 2 in Fig. 14 a and b. The addition of Ringer's solution alone in the amount of 1 cc does not produce a contraction. Test 6 in Fig. 14 provides the only meagre suspicion of BCh hydrolysis and thus of the presence of non-specific ChE. In that test, the beginning, *i.e.*, contraction 5, gave a column that was higher than the BCh control by an amount that may be considered to correspond to the effect of 0.5 cc of aq.h. The difference in height disappears during 2 hours' incubation. Since in the corresponding physostigmine-containing control there occurred no levelling of nearly the same magnitude, it seems reasonable to suspect that the enzyme contributed to this result. The manner of contraction shows a further difference in that the test fluid which contains aq.h. produces immediately, during the first minute (small notch in the columns), a marked contraction, which is then followed by a slower contraction evidently produced by BCh. Since even the contraction due to ACh is usually not very rapid it is possible that that part of the contraction which is ascribable to aq.h. is caused by some substance other than ACh.

Tests on the Specificity of Cholinesterase in Rabbit Aqueous Humour. — Table 11 shows the results of random tests with rabbit aq.h. samples drawn under greatly different conditions. These results again are only indicative of the type of ChE in the aq.h. In test 1 it would seem that even primary aq.h. was probably able to effect a slight hydrolysis of BCh, and it thus appears possible that the esterase in also primary aq.h. is non-specific, in similarity to the secondary aq.h. in test 2 (Fig. 15). There is no doubt but that hydrolysis of BCh had occurred in test 2. The amount of aq.h. was no greater than 0.2 cc but apparently it had become infected after the previous day's puncture, since it appeared turbid. The amount of 5 μg of BCh therefore does not seem excessively large for use also in the other tests.

TABLE 11

EXPERIMENTS ON SPECIFICITY OF CHOLINESTERASE IN RABBIT AQUEOUS HUMOUR

Test No.	Aq.h. per Test, cc	Substrate		Hydrolysis of Substrate, μg						ChE	Non-Specific ChE	Remarks
		ACh, μg	BCh, μg	1 h.	1 h. 15 min.	1 h. 30 min.	2 h.	2 h. 30 min.	3 h.			
1	0.3		5				+				+	Primary of 2 rabbits
2	0.075	0.05		+?			+			+		Primary of 1 rabbit
	0.2		5			+		5			+	Secondary of 2 rabbits
3	0.075	0.05				0.02			0.03	+		Primary of 1 rabbit
	0.2		5	—				—			—	Tertiary of 3 rabbits
4	0.05	0.02		0.01						+		ChE provoked
	0.3		2	+				+			+	with Mintacol; »primary« of 5 rabbits
5	0.05	0.02	+			< 0.01		> 0.01		+		ChE provoked
	0.3		2		+?			+?			+?	with HETP; »primary« of 3 rabbits

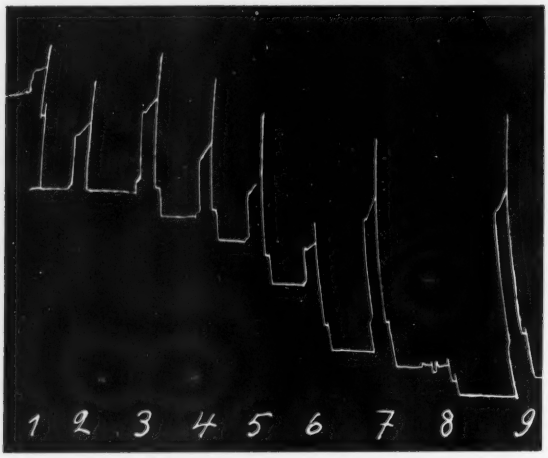
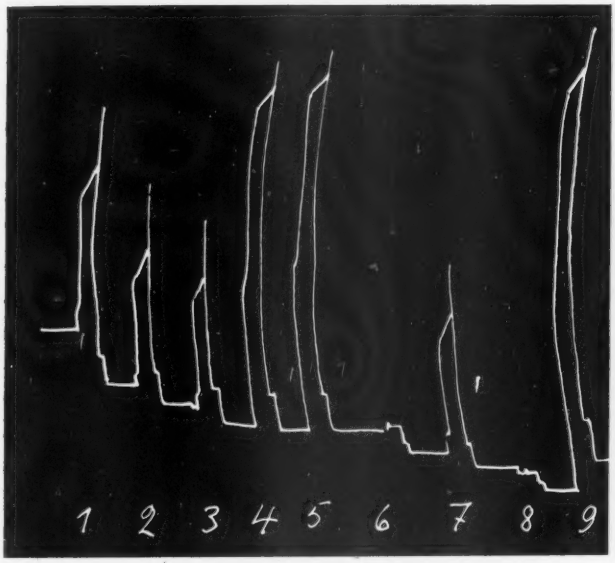


Fig. 15. — Effect of rabbit secondary aq.h. on BCh. 1, 2 and 5 = 5 μg BCh; 3 = 0.2 cc secondary rabbit aq.h. + 5 μg BCh; 6 = 3 incubated 1½ hrs.; 8 = 3 incubated 2½ hrs.; 4 = 0.2 cc secondary rabbit aq.h. + 5 μg BCh + 0.1 cc physostigmine salicylate 1: 1,000; 7 = 4 incubated 1½ hrs.; 9 = 4 incubated 2 hrs. 20 min. Test object leech muscle.



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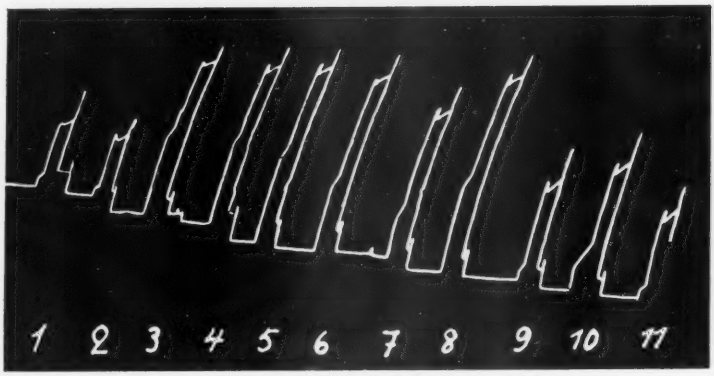


b

Fig. 16. — Effect of cat primary aq.h. on (a) BCh, and (b) on ACh. a) 1, 2 and 7 = 5 μg BCh; 3 = 0.3 cc cat aq.h. + 5 μg BCh; 4, 5 and 9 = 0.05 μg ACh; 6 = 3 incubated 1 ½ hrs.; 8 = 3 incubated 2 hrs. 15 min.

b) 1 and 2 = 5 μg BCh; 3, 6 and 8 = 0.05 μg ACh; 4 = 0.05 cc cat aq.h. + 0.05 μg ACh; 7 = 4 incubated 1 ½ hrs.; 11 = 4 incubated 3 ½ hrs.; 5 = 0.1 cc same aq.h. + 0.05 μg ACh; 9 = 5 incubated 2 hrs.; 10 = 0.03 μg ACh. Test object leech muscle.

Tests 4 and 5 indicate that at least a part of the artificially increased ChE in »primary» aq.h. is non-specific ChE. The contractions shown by the curves are by no means faultless, but it is possible to draw certain cautious conclusions.

Tests on the Specificity of Cholinesterase in Cat Aqueous Humour. — Because of the large amount of aq.h. in the cat eye, the cat would be very suitable for these tests, but the great tendency of hemorrhage into the aq.h. prevents its use. However, since the cat aq.h. was found to hydrolyse ACh rather strongly, it was considered justified to make an attempt to determine also the type of its ChE.

The results were already listed in Table 3, page 44 since the tests were made with primary aq.h. Tests 2, 3, 4, 5, 6 and 10 show definite hydrolysis of BCh. The possibility that blood was a contributing factor was dealt with in the same connection. However, the positive results cannot all be ascribed to the presence of blood in aq.h., since the Wagner test was negative (Fig. 16 a, test 10) and since one-sixth of the employed amount of this aq.h. was capable of hydrolysing only one-half of 0.05 μg of ACh in 3½ hrs., and one-third of the amount hydrolysed the same amount of ACh in 2 hrs. (Fig. 16 b), which corresponds to the usual hydrolysing action of aq.h. on ACh.

Only one test with cat aq.h. on mouse intestine appeared to be reliable; this is test 12 in Table 3. The result offers some small confirmation of the above mentioned observation that the ChE in primary cat aq.h. — or at least a dominant proportion of it — is non-specific in type.

DISCUSSION

Since the aq.h. and its proteins are derived from the blood and since the blood contains ChE, there is reason to assume that this enzyme is also present in aq.h. The tests described above offer consistent evidence that ChE can be demonstrated in almost all cases in the aq.h. of man and some other mammals, although it is present in minute amounts only. However, the method employed was capable of demonstrating that even this small amount is so

greatly variable that the ChE-negative results may reasonably be presumed to have been negative only because the ChE amount in those cases was outside the lowest limit of sensitivity of the technique.

Uvnäs and Wolff (1938) assumed that if the a.ch., especially that of the rabbit, is emptied too completely, ChE will become diffused into the a.ch. from the vitreous humour. Their opinion seems to be contradicted by the fact that in the normal eye the vitreous body is separated by a membrane, and it is hardly presumable that significant quantities of ChE would be able to permeate through this membrane during an aspiration procedure lasting a few seconds only. This opinion also seems to lack theoretic ground in regard to certain species of animal, since the ChE activity of the vitreous humour of, for instance, the horse is, strange to say, smaller than that of its aq.h. (Brückner 1943 a). According to de Roetth (1950) there is no ChE activity in either the vitreous humour or the aq.h. of the rabbit. In the present investigation, also, the amount of aq.h. aspirated was found to have no decisive effect on its ChE positivity or on the amount of ChE, for the highest degrees of ChE activity had a quite indefinite correlation to the amount of aq.h. — Evidence against the possible significance of the vitreous humour in this connection was also given by the exceptional test from the human eye (No. 7, page 40), in which, regardless of the obvious presence of vitreous humour in the aq.h. sample, the ChE activity did not differ from that shown by the other tests. It is true that the investigations of Uvnäs and Wolff gave evidence of an increased amount of ChE in secondary aq.h., but negative reactions of the primary aq.h. were not necessarily true results but may have been due to the inadequate sensitivity of the test method. The amount of substrate used by these workers (80 μg of ACh per 0.1 cc of aq.h.) was notably large.

In the opinion of some workers (Weve and Fischer 1939), withdrawal of the aq.h. sample by aspiration is unsuitable for this purpose. It is the writer's opinion that this technique holds its ground well against, for instance, spontaneous outflow. Because of the longer time required in the latter case, diffusion from surrounding tissues may actually become significant, for it is known that one-half of the volume of aq.h. is re-formed already during the first 3 min. (Kinsey and Grant 1944). Furthermore, in the

case of the restive rabbit the use of the more rapid method seems to be the only possible procedure to avoid trauma to the surrounding tissues.

Velhagen (1930) already stressed the significant part played by anaesthetics in the examination of intraocular fluids. In view of demands of the present work, the use of cocaine was considered simpler than that of pantocaine, for if it could be assumed that the anaesthetic is absorbed into the a.ch. already in 3—4 min., the effect of cocaine need only be taken into consideration as an inhibitor of ChE, both cocaine and pantocaine being known to be anticholinesterases (AntiChE:s). Pantocaine also produces hyperaemia of the conjunctiva and increases the proteins in secondary aq.h., but in primary aq.h. the protein levels are the same following the administration of either anaesthetic (Kronfeld and Lin 1936). Taking also into consideration that pantocaine has a considerably greater AntiChE action than cocaine, it does not appear probable that the anaesthetics used increased the ChE activity under these test conditions. This is borne out also by the similar results obtained without anaesthetisation from slaughter animals. It may be possible, however, that the anaesthetics played some part in the ChE-negative results.

The experiments of Arató and Arató (1952) are evidence that the large amount of aq.h. withdrawn by puncture (0.2—0.3 cc) alone is unable to give ChE positive results if the method is not sufficiently sensitive. In their tests the ChE-inhibiting action of the anaesthetic was the smallest possible, as cocaine was applied only to the points of fixation and penetration, yet ChE-negative results were obtained.

Abdon and Uvnäs (1937) were already aware of the effect of a high enzyme and low substrate concentration, and *vice versa.* Augustinsson (1948, 1949) expressed the opinion that the activity of the enzyme depends on the substrate concentration. The use of different concentrations of the substrate gave a typical bell-shaped curve, indicating that excessively high substrate concentrations inhibit esterase activity. This circumstance could not be observed in the present work, as the amount of substrate to be employed was ruled by the biological test object used. The ChE—substrate relationship can hardly have been essentially wrong, as the work was carried out under biological conditions, both enzyme and

substrate being of low concentration. There was very little possibility to vary the enzyme concentration, for the aim was to procure the result for each animal or patient individually, and diminishing of the originally weak ChE activity could not be considered.

The aq.h.—ACh relationship used by Brückner (1943 a) was approximately the same as employed in the tests in this investigation. His amount of physostigmine, on the other hand, was one-tenth of that used by the writer and caused inhibition of 80—75 per cent of the ChE at +37° C.

It seems evident that the aq.h. contains ChE irrespective of the species of animal. The earlier observations on this point have been supplemented by the finding in the present work that the aq.h. of the pig and the sheep are also ChE-positive. Velhagen (1932) found that aq.h. is capable of inhibiting the vagotropic action of the bovine uvea and retina, which is indirect evidence of the presence of ChE in aq.h. His test conditions were doubtlessly extremely demanding in order to demonstrate such weak ChE activity. However, it seems somewhat strange that aq.h. is mentioned as inhibitor of vagotropic properties in the same category with alkalis and blood, which have a considerably stronger inhibitive action.

Weve and Fischer (1939) suggested that the occurrence of ChE in the subretinal fluid is one of the proofs of the dissimilar origins of this fluid and aq.h. According to the studies then made, proteinase, the only enzyme they found in aq.h., was not present in the subretinal fluid. In the light of the writer's studies, at least this reason for difference in the origin of these fluids seems to be erroneous.

A great deal of study has been given to the origin of the ChE in aq.h. It would seem logical that this protein, like the others in aq.h., is derived from the blood in conformity with the theory of filtration and secretion (Kinsey and Grant 1944). If Brückner (1943 a) were correct in his finding that the aq.h. of slaughtered cows and horses contains, in relation to the total proteins, more ChE than the blood, we should have to presume that ChE penetrates the blood—a.ch. barrier more readily than other proteins, unless it is derived from entirely other sources. However, the very minute ChE values obtained in this work do not confirm the existence of such a disproportion between the ChE contents of serum and aq.h.

Although it was not one of the objects of this investigation to study whether the subject of ChE can throw any light on the etiology of glaucoma, it must be mentioned that reports in the literature give in this connection very conflicting ChE contents in the serum and the aq.h. This divergence of the findings is evidence of either one of two facts, *i.e.*, that no conclusive differences exist in the ChE activity of the serum and aq.h. of persons with glaucoma and with normal eyes, or that such differences are difficult to demonstrate by the methods available at present. Study of the possible absence of ChE in the glaucomatous eye would require a very large material in order to distinguish with certainty the deficiency from the minute normal amounts; conversely, in order to be clearly demonstrable, the increase in the ChE should preferably be in amounts several times the physiological amount.

The CO_2 values reported by Matteucci (1947), which were 0—0.8 cu mm in the aq.h. of glaucomatous patients, correspond approximately to the ChE amounts observed in this investigation, or, in units, to 0—0.0051 ChE unit (based on the formula that 1 mg of ACh at +37° C will yield 156 cu mm of CO_2). It remains to be determined if this amount is to be considered a deviation from the 0 values obtained for the non-glaucomatous aq.h. The series of cases examined by Matteucci — 12 glaucomatous and 14 non-glaucomatous subjects — was a rather small one.

Assuming that the aq.h. originates from the serum, and as demonstration even of minute amounts of ChE in aq.h. has been possible in the present investigation, a certain degree of doubt must be entertained regarding the findings of Gallois and Herschberg (1946), who, regardless of the increased ChE content of the serum, were able to demonstrate no ChE in the aq.h. of the glaucomatous eye.

A situation where the aq.h. of the glaucomatous eye would have a lower ChE content than the normal eye appears improbable, for should a normal amount of ACh be formed, the situation would only be a favourable one from the point of intraocular pressure. Furthermore, although the protein levels of aq.h. are normal in pure, compensated primary glaucoma (Kronfeld 1941), the highest protein levels have been found in the hydrophthalmic eye and in secondary and acute glaucoma (Francheschetti and Wieland 1928). The presence of more ChE would therefore also be presumable,

provided that the ChE—protein relationship moves parallel. However, if the ACh metabolism is changed completely, so that also less ACh is formed, the ChE activity may also be presumed to be weaker. We are here confronted with the question why anticholinesterases have a beneficial effect on the glaucomatous eye if the object of their action already is weaker than normal. A pathological increase of ChE in the glaucomatous eye would thus seem theoretically more plausible. Or is it probable that the AntiChE action in glaucoma does not consist in a reduction of the ChE content of all tissues of the eye?

The studies above described showing increased ChE activity in secondary aq.h. supplement the relatively few investigations earlier made on the subject (Uvnäs and Wolff 1938, Brückner 1943 a, Jaffe 1948, de Roetth 1950, Toda 1952). The increased ChE activity in secondary aq.h. appears logical in consideration of the protein content of the regenerated plasmoid aq.h. The protein content of, for instance, human aq.h. is normally 1—34 mg per 100 cc (Alaerts 1948, Süllmann 1951, Niedermeier 1952), and it is in creasedin the secondary aq.h. of the normal human eye *ad* 1,000 mg per 100 cc. In the rabbit eye it increases to as much as one-half of the amount of serum proteins, or *ad* 3,580 mg per 100 cc (Niedermeier 1952).

As stated previously, the aq.h. is very rapidly re-formed without regard to the anaesthetic used (Kronfeld and Lin 1936). No changes in the protein content have been found in samples drawn 10—60 min. after the primary puncture (Kronfeld, Lin and Luo 1941), but the amount of aq.h. withdrawn in the primary puncture and the anaesthetic used have a marked effect on the protein content of the secondary aq.h. Toda (1952) drew secondary samples after intervals of various length starting with 15 min. and observed a rapid disappearance of the ChE activity already during the first 4 hrs. In the present work, longer intervals were used in order to permit abatement of possible immediate effects of the anaesthetic and of other factors. Even after an interval of 4—5 hrs., and sometimes of 48 hrs., the ChE activity of the aq.h. was found to be definitely above normal. This serves to indicate how slowly balance is regained in the composition of aq.h. regardless of the rapid regeneration and circulation of the fluid. According to Kronfeld (1941), 2—3 weeks are required before the protein content of reformed aq.h. humour regains its normal level.

Regarding the type of ChE in aq.h., the results obtained in the present investigation demonstrate, in conformity with those of Jaffe (1948) and Toda, that the ChE in secondary aq.h. is non-specific in type. It also appears probable that the ChE in primary aq.h. (of the rabbit and the cat) is non-specific at least for the greater part. This might indeed be expected, but it does not seem to have been demonstrated experimentally earlier. De Roetth (1951), it is true, carried out extensive studies on this subject but he concluded that the primary aq.h. of the cat has no ChE activity. His samples were drawn under pentobarbital anaesthesia and tested by the colorimetric method, the sensitivity of which is as low as that of Warburg's method. On the other hand, secondary aq.h. drawn 20 min. later showed an activity typical of non-specific ChE and similar to that of cat serum. He found specific ChE in the primary aq.h. of the ox and in rabbit serum. The results of his tests with horse aq.h. were so unreliable that he disregarded them entirely. The ChE activity of the ox serum was so weak on all substrates that it led him to assume that the ChE in the aq.h. of this animal is derived by diffusion from the iris and the ciliary body. No attempt was made by de Roetth to study the type of ChE in human aq.h.

Explanations were provided, it is true, by de Roetth for the highly variable and unsystematic occurrence of the two types of ChE, but they seem rather farfetched and adapted according to the case. A common enzyme of the organism, such as ChE, would seem to call for greater uniformity in the explanation of its presence. Furthermore, if we adhere to the opinion that at least secondary aq.h. is derived from the serum, it would appear strange that the ChE in the rabbit serum is specific. The observations of de Roetth are compatible with the opinion that the aq.h. of man and all species of animal contains both specific and non-specific ChE but only the dominant type becomes manifest. On the basis of the present investigation it appears probable that both primary and secondary aq.h. contain chiefly non-specific ChE.

PART II

ANTICHOLINESTERASES

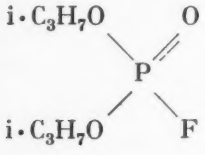
Anticholinesterases (AntiChE:s) belong to the group of autonomic drugs. The mechanism of their action may be understood on the basis of the theory of nerve impulse transmission presented by Loewi (1921). Fully independent of their own pharmacological action, AntiChE:s enhance cholinergic effects chiefly by protecting acetylcholine (ACh) against the hydrolysing action of cholinesterase (ChE). The numerous substances which inhibit ChE include a large number of non-related substances, of which Augustinsson (1948) has given a comprehensive list. The principal groups are that containing physostigmine, synthetic neostigmine and other urethanes, and the organic phosphoric compounds group. Physostigmine is the classical best known and powerful AntiChE. AntiChE:s are divided according to their mode of action into substances which reversibly combine with ChE, like physostigmine, and those that do it irreversibly, like polyalkylphosphates.

HISTORY OF POLYALKYLPHOSPHATES

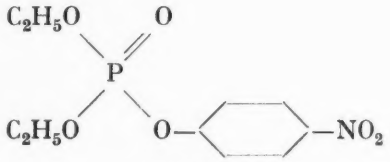
A number of synthetically prepared polyalkylphosphates have been known for a long time (de Clermont 1854, Nylen 1930). Their great toxic effect was observed by Lange and Krueger (1932), but it was first through the studies of Adrian, Kilby and Kilby (1940) and Adrian, Feldberg, Kilby and Kilby (1941) that this action was known to be based on AntiChE substances. The study of polyalkylphosphates progressed secretly in both Germany and Great Britain at the same time, and therefore a chronological

review of the results is difficult. In 1941 McCombie and Saunders synthesised DFP (diisopropyl fluorophosphate), and Adrian, Kilby and Kilby (1942) and Dixon and Macworth (1942) found that it possessed a still stronger inhibitive action on ChE than the dimethylesters previously developed. The ChE-inhibiting action of fluorides had been observed already in 1930 (Matthes, Plattner and Hintner b). In 1944 Schrader (Schrader 1950) developed p-nitrophenyl diethylphosphate (Mintacol), and DuBois and Mangun (1947) studied the antiChE activity of tetraethyl pyrophosphate (TEPP) and hexaethyl tetraphosphate (HETP). Work on dimethylamido-ethoxyphosphoryl cyanide (Tabun), which had been synthesised 1937 by Schrader in Germany (Bonnaud 1948), was continued by Holmstedt (1951).

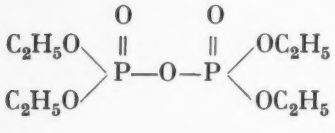
The formulas for polyalkylphosphates used in this work are as follows:



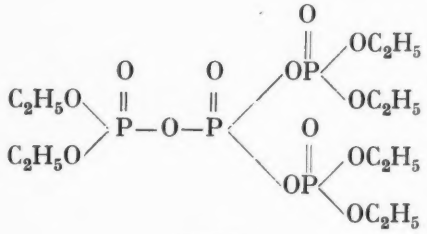
DFP
Diisopropyl fluorophosphate



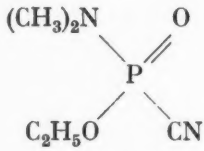
Mintacol
p-nitrophenyl diethylphosphate



TEPP
Tetraethyl pyrophosphate



HETP
Hexaethyl tetraphosphate



Tabun
Dimethylamido-ethoxyphosphoryl cyanide

CHEMICAL AND PHYSICAL PROPERTIES OF POLYALKYLPHOSPHATES

Polyalkylphosphates are colourless or light or dark brown oily liquids. They are either odourless or slightly aromatic (Forssling 1948). They are usually soluble in water and fat solvents but are also readily decomposed in water, with consequent loss of activity (Comroe, Todd and Koelle 1946, Dayrit, Manry and Seevers 1948, Grant 1950). An exception is Mintacol, which completely retains its activity for one year in an aqueous solution of pH 6.8 (Wirth 1949) but has been found to be readily hydrolysed in an alkaline solution (Augustinsson 1953 a). Polyalkylphosphates remain stable in arachis oil, absolute alcohol, toluene and xylene (Koelle and Gilman 1946, Weekers 1947, Brauer 1948). It has been observed that the less stable the compound, the stronger its inhibition of ChE (Holmstedt 1951, Augustinsson 1952, Aldridge and Davison 1952). However, the effect of DFP is approximately the same when instilled into the eye in aqueous or oily solution.

Polyalkylphosphates are heavier than water; their freezing point is from —40 to —50° C. and boiling point from +150 to +183° C.

MECHANISM OF THE ACTION OF ANTICHOLINESTERASES

As stated, the inhibition of ChE:s by AntiChE:s may be either reversible or irreversible. However, it is not easy to draw a demarcation line, for some substances probably inhibit in both ways. Aldridge (1950) has presented a formula for the theory and reaction of the entire AntiChE mechanism, as follows:

$$\underset{\text{Enzyme}}{E} + \underset{\text{Inhibitor}}{I} \overset{(1)}{\rightleftarrows} \underset{\text{Reversible enzyme inhibitor complex}}{(EI)^R} \overset{(2)}{\longrightarrow} \underset{\text{Irreversibly inhibited enzyme}}{(E)^I}$$

In the case of TEPP the stability of the phosphorylated enzyme probably is not very great and the TEPP interaction seems to be of a reversible nature after a given length of time (Bergel 1951). Opinions differ on this point (Brauer 1948, Augustinsson and Nachmansohn 1949 a), but Grob and Harvey (1949), Grob (1950) and Aldridge and Davison (1952) have stated that the inhibition of ChE by TEPP is not fully irreversible. On the other hand, HETP produces a practically irreversible inhibition of ChE *in vitro*,

although tests *in vivo* indicate that the combination is to a marked extent labile. It permits release of ChE in such amounts that the animal may recover within a short time (Dayrit, Manry and Seevers 1948). For this reason HETP occupies an intermediary position between DFP, which has an indisputably irreversible action (Comroe, Todd and Koelle 1946), and neostigmine. It would appear that the alkyl group of phosphatic AntiChE:s has a profound effect on the phosphorylation process (Saunders and Stacey 1948). Roughly grouped, the earlier known AntiChE:s — physostigmine and neostigmine — are classified as reversible inhibitors, and the polyalkylphosphates as irreversible.

RELATIONSHIP OF ANTICHOLINESTERASES TO SPECIFIC AND NON-SPECIFIC CHOLINESTERASE

When the existence of a specific and a non-specific ChE (Mendel and Rudney 1943 a) was known (cf. page 15), it was possible to demonstrate that reversible AntiChE:s have a stronger inactivating action on the specific ChE present in red cells and nervous tissue, whereas polyalkylphosphates, with the exception of Tabun (Holmstedt 1951, 1954, Augustinsson 1952), have a definitely greater affinity for non-specific ChE, which is found especially in serum or plasma (Mendel and Rudney 1943 a, Koelle and Gilman 1946, Hawkins and Mendel 1947, Augustinsson 1948, Grob 1950).

Non-specific ChE is about 100 times as sensitive to the inactivating action of DFP as specific ChE (Mazur and Bodansky 1946, Hawkins and Mendel 1947). Thus toxic symptoms are produced in the rabbit by relatively small amounts of DFP, since, according to the experiments of Hawkins and Mendel, specific ChE predominates in the plasma of this animal.

GENERAL PHARMACOLOGIC EFFECTS OF ANTICHOLINESTERASES

The effects of AntiChE:s are based on the inhibition of ChE and they are thus similar to the action of ACh. The effects of ACh, again, are similar to those of muscarine, on the one hand, and nicotine, on the other hand. The muscarine-like effects are parasympathomimetic, exciting the glands and the greater part

of smooth muscles. This action is abolished by atropine (Grob *et al.* 1947). The nicotine-like action stimulates the voluntary muscle fibers, autonomic ganglia and the secretion of adrenaline by the medulla of the suprarenal gland. Depending on the species of animal, muscarine- or nicotine-like effects may predominate in toxic states (Koelle and Gilman 1946, Forssling 1948). Generally speaking, muscarine-like effects are dominant after small doses and nicotine-like effects after large doses.

Thirdly, ACh administered in moderate doses stimulates the central nervous system. This effect is seen more clearly after polyalkylphosphates than after physostigmine and neostigmine, probably because of the greater solubility of polyalkylphosphates in fat solvents (Koelle and Gilman 1949, Chennels and Wright 1947). In large doses, ACh has the opposite effect and blocks the ganglia, thus reversing its own action.

In cases of AntiChE intoxication, death is probably due partly to paralysis of the respiratory centre and partly to bronchoconstriction (Kilby and Kilby 1947). Burgen, Keele and Slome (1949) believed that atropine does not inhibit the action of AntiChE:s on the central nervous system, but opinions to the contrary have also been advanced (Wescoe *et al.* 1948).

Dimethylamido-isopropoxyphosphoryl cyanide is the most potent of all known AntiChE substances (Holmstedt 1951), and it is generally accepted that Tabun is the strongest of those used in the present investigation. DFP is $5\frac{1}{2}$ times as strong as physostigmine and its inactivating action on ChE is already seen in a concentration of 10^{-10}, while physostigmine almost completely inhibits ChE at 8×10^{-7} (Dixon and Macworth 1942, Adrian, Feldberg and Kilby 1947, Burn 1952). According to Salerno and Coon (1949), the order of potency of these AntiChE:s, listed from the strongest to the weakest, is DFP > HETP > TEPP > physostigmine. However, DuBois and Mangun (1947), Mangun and DuBois (1947), Grob and Harvey (1949), and Augustinsson and Nachmansohn (1949 a) considered TEPP even stronger than DFP. In the opinion of Burgen, Keele and Slome (1949) TEPP and physostigmine are of equal strength and superior to HETP. Gautrelet and Scheiner (1939) considered that physostigmine and neostigmine have equal AntiChE properties, and Augustinsson (1948), in his comprehensive table of AntiChE substances, placed physostigmine and neostigmine with

DFP and HETP in the same class of strongest AntiChE:s (+++). Mintacol is 10 times as powerful as physostigmine in inhibiting the ChE of horse serum (Wirth 1949).

These slightly conflicting findings are not surprising in view of the greatly variable test conditions, especially in the case of experiments *in vitro* and *in vivo*.

It may also be pointed out that ChE may in turn hydrolyse AntiChE, which was the observation made by Aldridge (1953) on the action of plasma ChE on Mintacol.

For already some decades, physostigmine has been employed in biological work to sensitise muscle tissue to ACh. This sensitising action is based chiefly on the inactivation of ChE, and the sensitisation effected is 500—1,000-fold (Kahane and Lévy 1937 b). A similar, although weaker, action is exerted by DFP and other AntiChE:s also (Miquel 1946, Burgen *et al.* 1947, Quilliam and Strong 1949).

In general, physostigmine and neostigmine sensitise muscular tissue to a number of substances, as for instance 2- to 3-fold to barium (Führer 1918 c, Vartiainen 1933) and to potassium (Vartiainen and Kostia 1937). They are also capable of sensitising to ACh such muscles from which the ChE has already been destroyed by DFP (Miquel 1946).

In addition to their sensitising action, AntiChE:s also have a direct action on muscles (Harnack and Witkowski, 1876, Kahane and Lévy 1937 c) but only when used in high concentrations of *c.* 10^{-3}—10^{-4} (Miquel 1946). The iris muscle is not directly affected by physostigmine (Anderson 1905) nor by DFP (Leopold and Comroe 1946 b). The effect of neostigmine as well as that of ACh on skeletal muscle is the same in the presence or absence of ChE (Riker and Wescoe 1946). Pharmacologically, neostigmine is classified with the choline esters.

Because of their ACh-like action, AntiChE:s are vasodilatory and increase capillary permeability (Duke-Elder 1932, Swan and Hart 1940, Adler 1953). However, it has been said of DFP that it has all the effects of cholinergic substances except the peripheral vasodilatory and hypotensive effects (Grob *et al.* 1947).

PROTECTION AGAINST ANTICHOLINESTERASES

On observing that DFP intoxication in the cat can be prevented by prophylactic administration of physostigmine, Koelle (1946) made *in vitro* studies of the effect of 20 different AntiChE:s on the irreversible inactivating power of DFP. He found that physostigmine and neostigmine have a considerable ability to protect ChE, whereas pilocarpine has a weak protective capacity, and ACh no property of this kind. The protection afforded by reversible AntiChE:s has also been demonstrated by several other investigators (Comroe, Todd and Koelle 1946, Koster 1946, Gilman and Gattell 1948, Quilliam and Strong 1949, Augustinsson and Nachmansohn 1949 a, Burgen 1949 b, Swan and Gehrsitz 1951). Contrary to the opinion of Koelle, the substrate, mainly ACh, is also capable of protecting against irreversible AntiChE (Augustinsson and Nachmansohn 1949 a, Burgen 1949 b, Bain 1949, Augustinsson 1953 b). When administered after DFP, physostigmine tends to increase the toxic effects (Koster 1946).

It may also be pointed out that a protective action against Tabun has been obtained with procaine, most clearly for plasma ChE (Augustinsson and Grahn 1952).

Augustinsson and Nachmansohn (1949 a) observed that the enzymatic hydrolysation of ACh in the presence of physostigmine or neostigmine attained the same balance whether incubation of enzyme with the inhibitor was carried out before or after the addition of ACh. In the case of DFP and TEPP, on the other hand, a definite protective action was seen if the ACh was added to the enzyme simultaneously with them.

The probable explanation for this protective phenomenon is that the protective substance combines with ChE, so that the latter is unable to receive AntiChE.

EFFECT OF ANTICHOLINESTERASES ON THE EYE

Even in toxic doses, AntiChE:s have but a slight effect on the eye when given by systemic administration (Dayrit, Manry and Seevers 1948, using HETP). Miosis is prominent in experimental animals only when lethal doses are given (Koelle and Gilman 1949). Intramuscular administration into man has no effect on miosis or accomodation of the eye (Leopold and Comroe 1948) nor is the eye

affected by injections or peroral administration of, for instance, Mintacol (Wirth 1949, Huerkamp and Wagner 1950). However, Mykalyan (1952) observed a lowered pressure also in the other eye after the subconjunctival injection of neostigmine.

Under normal conditions, substances instilled into the eye are brought by way of the lacrymal passages onto the nasal mucosa, where they are rapidly absorbed. If the instilled substance produces a strong irritation in the conjunctiva, the greater part may be washed away by the lacrymal flow. Several investigators (Grant 1948, Thiel 1949, Knüpffer 1949, Huerkamp and Wagner 1950) have been unable to observe any systemic symptoms following the instillation of polyalkylphosphates into the eye in the usual doses. However, there are also several reports of definite systemic effects. Thus, toxic symptoms have been produced by instillation of physostigmine into the eye at intervals of 10 min. (Sugar 1951), and DFP has brought about changes in the ChE activity of the plasma or serum of man, cat and dog (Leopold and Comroe 1946 a, b). One drop of 4 per cent TEPP (Marr and Grob 1950, rabbits) and two drops of 1 per cent DFP in each eye (Leopold and Comroe 1946 b, cats and dogs) produced salivation and muscle tremors. Instillation of DFP caused nervous symptoms in children (Abraham 1953). A slight reduction in the serum ChE level, as a sign of systemic absorption, has also been observed after instillation of DFP into glaucomatous and normal eyes (Leopold and Comroe 1946 b). A 5 per cent solution of neostigmine has frequently caused toxic symptoms, in sensitive persons even vomiting (Rosengren 1943).

DFP instilled into the eye probably presents the strongest local effects of AntiChE:s. Such effects are miosis, ciliary spasm, lacrymal flow, false myopia, headache, and changes in intraocular pressure (Leopold and Comroe 1946 b, Scholz and Wallen 1946, Ferrer 1950). The observation that the effect of DFP is of longer duration in the normal than in the glaucomatous eye points to a general disturbance in the ACh—ChE system in glaucoma (Leopold and Comroe 1946 a, Wheeler 1950).

Miosis and Accommodation Spasm. — Physostigmine and neostigmine as well as polyalkylphosphates, when instilled in the eye, are capable of producing the maximum contraction of the pupil.

Only the rapidity and duration of the effect are variable. Grant (1948) studied the threshold value for miosis and found it to be identical for physostigmine, DFP and TEPP, at a concentration of 0.001—0.005 per cent. According to Holmstedt (1951), Tabun has the same threshold value. However, 0.05 per cent of DFP is required to produce maximum miosis (Leopold and Comroe 1946 a, b), 0.25 per cent of HETP to produce it in 5 min. (Dayrit, Manry and Seevers 1948), and 1 per cent of HETP to effect it in a few minutes (Burgen, Keele and Slome 1949). The effect of physostigmine is equally rapid but of shorter duration. According to the comparative studies of Linn and Tomarelli (1952), only the miosis produced by DFP lasts definitely longer (45 hrs.) than that seen after other substances.

The contracting action of physostigmine on the atropinised pupil was observed already by Argyll Robertson in 1863. It was confirmed by Jackson (1950), whereas Linn and Tomarelli were unable to find this effect.

The effect on accommodation does not parallel the miosis. Thus, for instance, in the experiments of Grant (1948), 0.01 per cent TEPP had as yet no appreciable effect on accommodation. In general, the duration of accommodation varies according to the substance and species of animal. After physostigmine and neostigmine it lasts for *c.* 24 hrs. and after polyalkylphosphates for several days. For instance, after DFP, miosis was found to begin in 5—10 min. and accommodation spasm in 1 hr. (Leopold and Comroe 1946 b). The former lasted 3—9 days and the latter 3—7 days (Scholz and Wallen 1946). A miosis lasting as long as 2½ months was obtained with large doses (0.6 mg) instilled into the eye (Modell *et al.* 1946).

A severe ciliary spasm causes headache, which is a symptom also seen after physostigmine (Eidelman 1951). However, it may often be prevented by starting the instillations with mild dilutions (Mayer 1948).

Effect on Blood Vessels. — Vasodilatation and increased capillary permeability are presumably one of the most important effects of AntiChE:s on the eye also. The most frequent findings are congestive iritis and ciliary and conjunctival congestion (Dunphy 1949), which is considerably more marked after polyalkylphosphates than after physostigmine and neostigmine. Gittler (1950),

it is true, found no irritation in the conjunctival blood vessels following Mintacol. The congestion may appear as late as 24—48 hrs. after application. Aldrige *et al.* (1947) ascribed this delay to the increased action of ACh. As the eye becomes accustomed to the substance the congestive symptoms usually disappear within 2—3 days. Atropinisation prevents the appearance of the congestive symptoms (Marr and Grob 1950, Linn and Tomarelli 1952).

As a result of the vascular changes, plasmoid aqueous with an elevated protein level is found in the a.ch. (Wessely 1913, Swan and Hart 1940, Scholz 1946, von Sallmann and Dillion 1947, Aldrige *et al.* 1947). Whereas the protein content of rabbit aq.h. is usually 40 mg per 100 cc (Adler 1953), von Sallmann and Dillion found values as high as 1,450 mg per 100 cc following the administration of a 0.2 per cent solution of DFP. Grant (1948) reported a case of glaucoma in which increased pressure was observed after TEPP, but 24 hrs. later the aq.h. was microscopically clear (no cells). Numerous experiments with fluorescein, inulin and other substances support the opinion that AntiChE:s increase vascular permeability (Wessely 1913, 1953, Leopold and Comroe 1946 b, von Sallmann and Dillion 1947, Bayo and Peña 1951, Miller and Swanljung 1951). Normally, already, the ciliary capillaries permit proteins to pass through somewhat more readily than other blood vessels of the eye, but this difference is still more convincingly brought out after DFP instillation (von Sallman and Dillion). Although the choroid vessels also participate in the formation of aq.h. (Vilstrup 1952), it has not been possible to demonstrate any change in their calibre as a result of autonomic drugs (Leopold 1951).

Iserle and Rezek (1951) injected into the a.ch. »Ts-219», a substance identical to Mintacol, and observed hyperaemia of the iris, haemorrhages and fibrinous exudation.

Effect on Intraocular Tension. — The purpose of AntiChE administration is to reduce intraocular pressure. For many decades physostigmine has been employed to obtain this effect. However, it has been known for already a long time that physostigmine (Wessely 1913, Linn and Tomarelli 1952) and neostigmine (Kull 1942, Brückner, Hermann and Jent-Peyer 1949), when instilled into the eye, usually produce a slight, although transient, increase in the intraocular pressure. A similar effect is seen still more clearly

after polyalkylphosphates both in test animals (von Sallmann and Dillion 1947) and in man (Scholz 1946, Scholz and Wallen 1946). As a result of contralateral pupillary dilatation, increased pressure may also be seen in the untreated eye (Linn and Tomarelli 1952). Evidence of the part played by vasodilatation in the increase in tension is also supplied by the findings of Bietti (1954) regarding the effect of nicotine and ACh in provocative tests of glaucoma.

However, a permanent increase in the pressure has also been caused frequently by AntiChE:s. Dunphy (1949) observed such an increase in one case following physostigmine, but numerous cases have been observed after polyalkylphosphates (Leopold and Comroe 1946 a, Leopold and McDonald 1948, von Sallmann and Dillion 1947, Grant 1948, Dunphy 1949, Hauck and Biggins 1949, Weinstein 1949, Gittler 1950, Stone 1950, Butler 1952, Zekman and Snydacker 1953). Cases of congestive and narrow angle glaucoma especially have a tendency to react with increased pressure, sometimes to such an extent that surgical intervention has been necessary.

In general, however, AntiChE:s reduce the intraocular pressure. Polyalkylphosphates have a stronger action than the substances earlier employed, as has been demonstrated in numerous tests with experimental animals and with persons with normal and glaucomatous eyes. According to studies with DFP (Leopold and Comroe 1946 a, b, McDonald 1946, Marr 1947, Haas 1948, Hughes 1948, Leopold and McDonald 1948, Dunphy 1949, Goedbloed 1949, Bond 1949, Raiford 1949, Weinstein 1949, Stone 1950, Tichomirov and Dmitrieva 1952), an advantageous effect has frequently been obtained in eyes in which several miotics earlier have failed. The hypotensive action of TEPP was observed by, among others, Grant (1950), although it did not prove superior to physostigmine in experiments by Marr and Grob (1950). Mintacol produces no significant decrease in the pressure of the healthy eye but has frequently proved valuable in glaucoma (Büning 1949, Glees and Wüstenberg 1949, Müller 1949, Thiel 1949, Huerkamp and Wagner 1950, Neuenschwander 1950, Iserle and Rezek 1951, Moreu Gonzalez-Pola 1951, Knüpffer 1952).

In the studies of Linn and Tomarelli (1952), the fall in pressure was inversely proportional to the symptoms of irritation. The degree of miosis has no correlation to the changes in permeability

(Swan and Hart 1940) nor to the fall in pressure (Stone 1950). A reduction of pressure can also be effected without miosis in eyes with previous iridectomy (Leopold and Comroe 1946 b). Dunphy (1949) has made a clear division of AntiChE action into factors which tend to decrease intraocular tension and those which increase it. The latter include vasodilatation, which tends to increase the volume of the iris and the ciliary body, and cyclotonia, causing the lens to push the iris root forward, compressing Schlemm's canal.

Results of uniform and systematic tests are not available for comparison of the potency relations of the different substances. The following results were reported by Linn and Tomarelli (1952), who compared the highest concentrations used in the treatment of glaucoma. The onset of miosis was equally rapid after physostigmine, DFP and TEPP, and slower after neostigmine (and stigminene), the two other substances tested. For duration of the miosis the order of the substances, listed from the strongest to the weakest, was DFP, TEPP, physostigmine. The greatest reduction in the pressure measured after 1 hr. was produced by neostigmine and TEPP, followed by DFP, whereas physostigmine caused an elevation in tension. In general, the effect of neostigmine is weaker than that of physostigmine (Sugar 1951). However, according to Kull (1942) and Simonelli (1947), it lasts longer than that of physostigmine and it is free of the untoward effects of the latter. For the treatment of glaucoma, Mintacol has been regarded as equal or somewhat superior to physostigmine (and pilocarpine) (*e.g.*, Glees and Wüstenberg 1949).

Effect on Tissues of the Eye. — Although AntiChE:s are readily absorbed by the eye, the corneal epithelium is not damaged by, for instance, TEPP even in the pure state (Grant 1948). No histological changes were found by Marr and Grob (1950), also, following the use of TEPP, nor by Leopold and Comroe (1946 b) even after direct injection of DFP into the a.ch. Scholz (1946), on the other hand, found epithelial blisters on the ciliary processes during the period of elevated tension in eyes instilled with DFP.

Physostigmine potentiates the action of certain anaesthetics on the cornea. Thus procaine, which otherwise has no anaesthetising effect on the eye when given by instillation, has this effect when

instilled with or after physostigmine. Greig, Holland and Lindvig (1950) ascribed this to changes in the permeability of the cornea due to inhibition of ChE, but Redi and Toxiri (1952) believed it to be entirely independent of AntiChE properties. Keaslin *et al.* (1951) observed that subcutaneous (?) injections of physostigmine caused partial anaesthesia of the cornea, which was not enhanced by the addition of procaine. They considered this phenomenon to be due to the muscarine-like effects of the physostigmine.

TOXICITY OF ANTICHOLINESTERASES

The toxicity of AntiChE:s is directly proportional to the inhibitive action of ChE *in vitro* (Holmstedt 1951) and it is dependent on a number of factors, such as solubility, velocity of hydrolysis and rate of ChE resynthesis. According to Augustinsson (1952), the toxicity is determined by the rate of volatility. The toxic symptoms are produced by an excess of ACh, the earliest symptoms being similar to the effects of muscarine (Grob, Garlick and Harvey 1950). A comparative estimation of the tests for toxicity is difficult because of the use of different test animals. A toxic effect is usually obtained with doses of tenth-parts of one milligram per kilogram of body weight. Some of the doses reported in the literature may be mentioned here. The lethal dose of DFP is 1.4 mg/kg instilled into the rabbit eye (Scholz 1946), and LD_{50} is 4 mg/kg injected subcutaneously into mice and 0.5—0.75 mg/kg given intravenously to rabbits (Kilby and Kilby 1947). The toxic dose of HETP for the cat is, at 0.2—0.3 mg/kg, of the order of physostigmine, and the lethal dose is 0.8—2.0 mg/kg (Burgen *et al.* 1947, Burgen, Keele and Slome 1949). TEPP is four times as toxic. DuBois and Mangun (1947) and Deichmann and Witherup (1947) have reported the toxic doses of HETP and TEPP for rats to be 1—3 mg/kg. For Mintacol, LD_{50} was *c.* 0.3—0.8 mg/kg given subcutaneously, intramuscularly or intravenously to the mouse, rat, guinea pig, rabbit and cat (Augustinsson 1953 b).

The first toxic symptoms are a dry throat and a feeling of tightness in the chest (Sollmann 1949). Several cases of intoxication and even of death from polyalkylphosphates have been reported among laboratory and factory workers (Wood 1950, Bidstrup 1950, Abrams, Hamblin and Marchand 1950, Sedan 1952).

PROPHYLAXIS AND THERAPY OF POLYALKYLPHOSPHATE POISONING

The protective properties of physostigmine, neostigmine and the choline esters were already discussed (page 73). Thus, polyalkylphosphate intoxication can frequently be prevented by first administering physostigmine. The previous administration of atropine is also able to inhibit the development of muscarine-like symptoms (Scholz 1946, Leopold and Comroe 1946 b) and to protect against a 3-fold lethal dose (Dayrit, Manry and Seevers 1948). However, because of the delayed onset of the toxic symptoms, atropine should not be administered as a prophylactic unless the patient can be held under observation for 24 hrs. Whether administered prophylactically or after the onset of intoxication, atropine abolishes the muscarine-like effects (Goedbloed 1949), like all parasympathomimetic blocking agents. But since it does not abolish the spasms produced by the nicotine-like effects, which are reversed in turn by the magnesium ion, simultaneous use of both of the above mentioned substances is the best antidote (McNamara, Koelle and Gilman 1946, Modell and Kropp 1946). In intoxications an hourly intramuscular dose of 2 mg of atropine has been recommended.

RESISTANCE, ACQUIRED TOLERANCE, AND ALLERGY

One of the drawbacks of the earlier known AntiChE:s is sometimes their gradual loss of effect after continued use (Rosengren 1943). The same is true of polyalkylphosphates (Bayo and Peña 1951), for already within a few months some patients have acquired tolerance for the hypotensive action of, for instance, DFP (Raiford 1949, Hauck and Biggins 1949, Ferrer 1950) and TEPP (Grant 1948). Leopold and Cleveland (1953) concluded that resistance to DFP develops within 6 months. A marked tolerance has also been found to develop for Mintacol (Wessely 1949). Huerkamp and Wagner (1950), in a series of 200 patients, found 14 patients resistant to Mintacol.

Allergy quite frequently makes it necessary to discontinue physostigmine therapy because of the development of a troublesome inflammation of the conjunctiva, presumably due to chemical irritation by the base. Similar sensibilisation to DFP has

been seen after administration of the drug for various periods (Leopold and McDonald 1948, Raiford 1949, Grant 1950, Stone 1950, Guerry 1951); however, Leopold and Cleveland (1953) consider this rather rare. Allergy to TEPP has been observed after ½—6 months' use (Grant 1948). These cases proved to be allergy to the AntiChE itself and not to the arachis oil, as sometimes is also the case.

RARE UNTOWARD EFFECTS

At the same time as it is important to know the advantageous properties applicable to clinical use, certain dangers connected with the instillation of AntiChE:s into the eye should not be disregarded. By producing a contraction in the musculature of the ciliary body, they also cause a shortening of the meridian fibres. In doing so they have a tendency to cause retinal detachment, which may result in ablation especially in the myopic eye. This is promoted also by transudation of plasma as a result of the congestion of the choroidal vessels (La Rocca 1952). Already in 1940, Gradle and Snydacker reported some cases of ablation in connection with the administration of physostigmine and pilocarpine. It is difficult to determine whether or not these complications are more common when polyalkylphosphates are used, but some cases are reported to have been caused by DFP (Marr 1947, Macrae 1948, Scheie 1949, Westsmith and Abernethy 1954) and at least one by Mintacol (Knüpffer 1952).

Cysts at the pupillary margin have been found not only after pilocarpine and physostigmine but also after polyalkylphosphates, for instance Mintacol and DFP in concentrations even as low as 0.005 per cent (Glees 1951, Abraham 1953, 1954, Swan 1954, Huber 1954).

The effect of variable doses of ACh, especially the tendency of large amounts to produce ganglial blocking, provides the explanation for many unexpected paradoxical effects (Leopold 1951 a, 1952). An example is mydriasis produced in cats with HETP and TEPP in intravenous doses of 0.4—0.6 mg/kg body weight (Burgen, Keele and Slome 1949).

An exceptional case is also that published by Dollfus (1948), in which paralysis of the third cranial nerve and mydriasis occurred after instillation of an 0.5 per cent solution of DFP.

ADVANTAGES OF POLYALKYLPHOSPHATES IN CLINICAL APPLICATION

Instillation at longer intervals and maintenance of an even pressure are probably the major advantages of the new AntiChE substances in those cases of glaucoma in which the hypotensive action of these substances is beneficial. Such forms of glaucoma have been found to be especially aphakic glaucoma (McDonald 1946, Lebensohn 1946, Schepens 1947, Leopold and McDonald 1948, Dunphy 1949, Stone 1950) and wide-angle glaucoma (Bond 1949). This prolonged effect gives the AntiChE:s, especially DFP and Mintacol, an important position in the treatment of accommodative strabismus also. DFP has proved its excellence as a miotic in round-pupil extraction of cataract and following cyclodialysis (Lebensohn 1946). Being powerful miotics, AntiChE:s are needed to rapidly overcome the mydriasis induced for examination (Goedbloed 1949, Böck and Veitl 1949, de Ocampo 1952). They frequently give relief in absolute glaucoma, not by their pressure-reducing action but because of their analgesic property (Mayer 1948, Stone 1950), and by rapidly decreasing the intraocular pressure they may make operation possible. They do not, however, alter the indications for surgical measures (Weekers 1947). Their use is exceptional for the improvement of vision in aphakic eyes by means of a small pupil (Rotter 1951).

Already in 1913 Wessely observed that the capability of calcium to reduce vascular permeability diminishes the protein content of aq.h. following instillation of physostigmine. When used with vaso-constrictors (epinephrine, synephrine, privine) or antihistamines, the field of usefulness of alkylphosphates in glaucoma is expanded further (Sugar 1947, 1951, von Sallmann and Dillion 1947).

SUMMARY OF THE LITERATURE

It is observed from the numerous studies on AntiChE:s that the ultimate effect of these substances is the result of several factors, no single factor alone being able to give a conception of the combined effect. Since ophthalmology is an important field of use of these substances and since they especially act as ChE-inhibitors, their effect on the ChE activity of the eye is of great interest. With this in view de Roetth (1951) studied the ChE

activity of the iris and the ciliary body of the rabbit eye after instillation of DFP and observed a definite reduction of activity. No corresponding investigations have been made of the aq.h.[1], there only being the statement that no appreciable increase in the ACh content of the aq.h. was seen after DFP instillation (von Sallmann 1950). For this reason the experiments described below were carried out.

[1] After this study was written, the author received a report of Sugawara (1953), who had observed inhibition of ChE activity in aq.h. by vagostigmine and DFP.

PRESENT INVESTIGATION

METHODS

The tests were carried out with seven AntiChE substances, among which physostigmine and neostigmine represented the earliest known group of AntiChE:s, and DFP, TEPP, HETP, Mintacol and Tabun the new polyalkylphosphate group. The determinations were made from rabbit aq.h. Using a normal dropper, a given amount of experimental substance was usually instilled into the rabbit's right eye. The concentrations and number of drops given in each test are listed in detail in the tables shown in connection with the results of each test group. The substance was allowed to act for a given length of time, which also is stated in each table, and the aq.h. sample was then withdrawn by puncture and aspiration, using the technique described in Part I. The sample was incubated with a known amount of substrate (ACh) at + 37° C and the hydrolysed amount of ACh was determined biologically, using m. rectus abdominis of the frog and dorsal muscle of the leech.

When a solvent other than water was used for the experimental substance, an equal number of drops of the solvent (arachis oil or propylene glycol) was instilled into the left eye (control). The aq.h. sample was drawn from the control eye immediately following withdrawal of the sample from the treated eye. In the course of the experiment the rapidity and magnitude of miosis and the possible symptoms of irritation were observed but are not stated in the tables.

Since ChE is present in aq.h. in very minute amounts only, and since its specificity is not always conclusively demonstrable, the only substrate employed was ACh, even though there would have been theoretic grounds also for the use of various other substrates in order to elucidate possible differences in the effect of physostigmine and neostigmine on the one hand and polyalkylphosphates on the other hand.

TABLE 12

EXPERIMENTS WITH PHYSOSTIGMINE

Test No.	Test Substance	Concentr., per cent	No. of Drops	Time of Action, h. min.	Amt. of aq.h. per Puncture, cc		ChE in Prim. aq.h. (Control)	Effect on ChE in aq.h.			Aq.h. per Test, cc	Hydrolysis of ACh, μg					Test Object
					R.E.	L.E.		Inactivated	No Change	Increased		30 min.	1 h.	1 h. 30 min.	2 h.	3 h.	
1	Ph.sa.	0.5	2	2	0.16	0.3	+	+			0.05		—		—		Frog
			2	40									(0.1)		(0.2)		
2	»	»	2	2 30	0.27	0.3	+	+			»		—		—		»
			2	1									(0.1)		(0.15)		
3	»	»	2	4	0.25	0.27	+	+			»	—?			0.1		
												(0.1)		(0.2)	(0.25)		»
4	»	»	2	2	0.13	0.3	+	+			»		+		0.3—0.4		»
													(0.1)		(0.4)		
5	Ph.su.	2.0	1	1 50	0.22	0.22	+	+			»		—			—	Leech
													(+)			(0.02)	
6	»	»	2	1 55	0.18	0.23	+		±		»	+			0.02		»
												(+?)			(0.03)		
7	»	5.0	1	45	0.21	0.25	+?	+			»		—		—		»
													(+)		(<0.005)		
8	»	»	»	2 10	0.21	0.22	+	+?	±		»	+		+			»
												(+)		(+)			
9	»	0.5	?	4 30	0.2	0.2	+ [1]	+			»	0.2		0.4			Frog
												(0.5)					
10	»	»	2	45	0.2	0.15	[2]	+?		—		—		—			Leech

[1] Secondary aq.h. from eye physostigminised immediately after first puncture.

[2] Primary aq.h. was not available, as eye had been treated with Mintacol.

The pupil of the physostigmine-treated right eye showed maximum miosis in all cases. Values in parentheses are for control eye. Amounts of ACh: frog muscle, 0.5 μg; leech back, 0.05 μg. Ph.sa. = physostigmine salicylate. Ph.su. = physostigmine sulphate.

EXPERIMENTS WITH PHYSOSTIGMINE

Table 12 shows the results obtained in the tests with aq.h. from eyes instilled with physostigmine. It is observed, in the first place, that quite independent of the manner and amount of instillation and time of action, physostigmine did not increase the ChE activity of aq.h. in any of the cases. On the contrary, it had in most cases a conclusively inactivating (Fig. 17, contraction 9

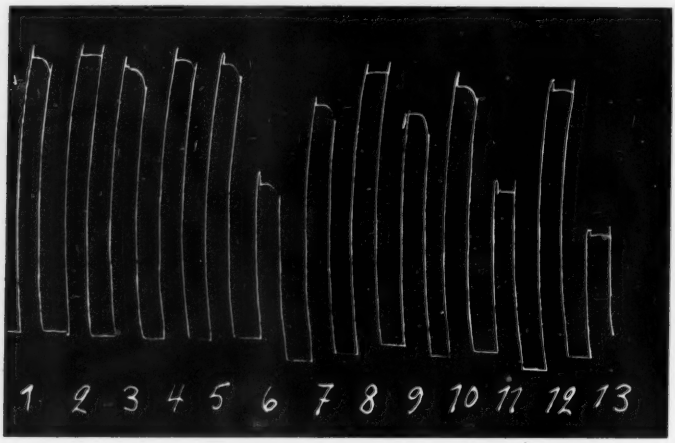


Fig. 17. — Effect of physostigmine on the ChE activity of aq.h. 2, 4, 8, 10 and 12 = 0.5 μg ACh; 1 = 0.5 μg ACh + 0.05 cc aq.h. from physostigminised eye; 5 = 0.5 μg ACh + 0.05 cc aq.h. from control eye; 3 = 1 incubated ½ hr.; 9 = 1 incubated 2 hrs.; 7 = 5 incubated ½ hr.; 11 = 5 incubated 1 ½ hrs.; 13 = 5 incubated 2 hrs. Test object frog rectus.

compared with control 13) and even a completely inactivating action (Fig. 18, contractions 5, 9, and 7, 11; also contractions 17, 21, 25, and 19, 23, 27). Complete inactivation signifies here that, within the limits of sensitivity of this method, no hydrolysis of ACh by aq.h. was observed.

The instilled amount of physostigmine used in test 8 was so great that its absorption clearly produced systemic toxic symptoms, for the rabbit became distinctly limp.

The amount of absorbed physostigmine probably plays a rôle in the ChE activity of aq.h., for in the two tests (Nos. 6 and 8) in which no change was seen in the ChE activity of aq.h. when compared with the control, the largest amounts of physostigmine

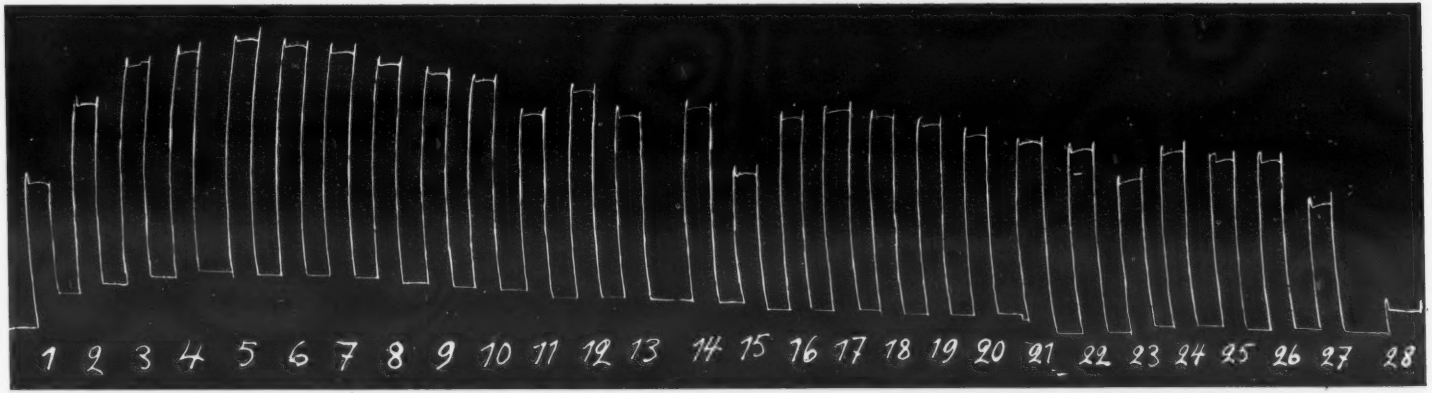


Fig. 18. — Effect of physostigmine on the ChE activity of aq.h. 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26 = 0.5 μg ACh; 5 = 0.5 μg ACh + 0.05 cc aq.h. from physostigminised eye; 7 = 0.5 μg ACh + 0.05 cc aq.h. from control eye; 9 = 5 incubated 1 hr.; 13 = 5 incubated 2 hrs. (only 0.47/0.5 cc of sample); 11 = 7 incubated 1 hr.; 15 = 7 incubated 2 hrs. (only 0.47/0.5 cc of sample); 28 = 7 incubated 3 hrs. 45 min.

17 = 0.5 μg ACh + 0.05 cc aq.h. from physostigminised eye of another rabbit; 21 = 17 incubated 1 hr.; 25 = 17 incubated 2 hrs.; 19 = 0.5 μg ACh + 0.05 cc aq.h. from control eye; 23 = 19 incubated 1 hr.; 27 = 19 incubated 2 hrs. Test object frog rectus.

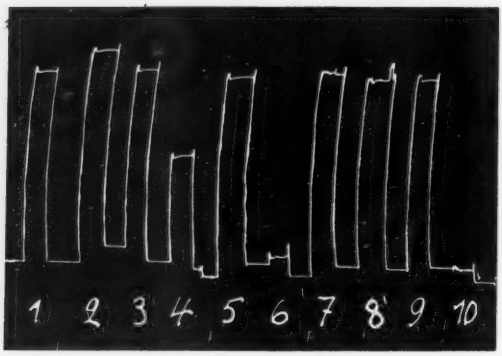


Fig. 19. — Effect of physostigmine on the ChE activity of secondary aq.h. 1, 3, 5, 7 and 9 = 0.5 μg ACh; 2 = 0.5 μg ACh + 0.05 cc secondary aq.h. from eye physostigminised after primary puncture; 4 = 2 incubated ½ hr.; 6 = 2 incubated 1 hr.; 8 = 0 5 μg ACh + 0.05 cc secondary aq.h.; 10 = 8 incubated ½ hr. Test object frog rectus.

and the longest times of action were used. In test 7 the time was only 45 min. and it is therefore possible that after 2 hours' action a similar doubtful (±) result might have been obtained.

In test 9 (Fig. 19) the physostigmine instillation was given immediately following the primary puncture and it was allowed to act for 4½ hrs. It would seem presumable that absorption of the substance by way of the puncture canal would be facilitated and that consequently a relatively large amount of physostigmine would enter the a.ch. This probably is indeed the case, for in this test 0.05 cc of aq.h. from the control eye hydrolysed 0.5 μg of ACh to 0 value in ½ hr. (contraction 10). The aq.h. of the physostigminised eye, on the other hand, caused a slight contraction even after 1 hour's incubation (contraction 6), the difference in the activity of the eyes thus being marked.

The amount of aq.h. obtained from rabbit eyes, and even from the eyes of the same rabbit, were variable, but no uniform correlation is seen in the table between the amount of aq.h. and the inactivation of ChE.

EXPERIMENTS WITH NEOSTIGMINE

The results obtained in the experiments with neostigmine are listed in Table 13. The same principal observation is made as in the

EXPERIMENTS WITH NEOSTIGMINE

Test No.	Test Substance	Concentr., per cent	No. of Drops	Time of Action, h. min.	Amt. of aq.h. per Puncture, cc		ChE in Prim. aq.h. (Control)	Effect on ChE in aq.h.			Aq.h. per Test, cc	Hydrolysis of ACh, μg					Test Object
					R.E.	L.E.		Inactivated	No Change	Increased		30 min.	1 h.	1 h. 30 min.	2 h.	3 h.	
1	Pr.ms.	3	1 1	2 1	(0.26)	0.2	+	+?	±		0.1			+ (Stronger +)			Frog
2	»	»	1 1	2 30 30	(0.26)	0.26	+	+			»			+? (Stronger +)			»
3[1]	»		1 1	2 2	(0.23)	0.28	+[1]	+			»		+ (0.3)				»
4[1]	»	»	2	1	0.3	(0.3)	+[1]		±	—	0.05		+ (+)		+ (+)		»
5[1]	»	»	2	4 30	0.15	(0.28)	+[1]	+			»			0.3 (0.5)		0.5	»
6[2]	»	»	2	20	(0.25)	0.15	+[2]	+			»		— (0.3)		+ (0.5)		»
7	»	»	1 1 1	3 50 2 50 50	(0.23)	0.22	+	+			0.1		— (0.1—0.2)				»
8	»	»	2 2 2	2 50 1 30 20	(0.33)	0.23	+	+			0.1		— (0.1)		(0.2)		»
9	»	»	2	2		0.25	[3]		±?		0.05	—?	+		0.01		Leech
10	»	»	2	1	0.25	(0.3)	[3]		±				+? (+?)		+ (0.1)		Frog
11	Pr.br.	10	1	1 35	0.23	(0.23)	—	+?		—	0.05	— (—)		— (—)			Leech
12	»	20	1	55	0.14	(0.23)	—	+?		—			— (—)				»
13	Pr.ms.	0.38 mg/1kg	1		(0.2)	0.23	+	+				— (+?)			+ (0.01)		»
14	»	0.25 mg/1kg	1	10	(0.25)	7.28	+?	+?		—	— (—)	—	— (—)	— (+)			»

[1] Secondary aq.h. [2] Tertiary aq.h. [3] Not tested.
Pr.ms. = prostigmine methylsulphate. Pr.br. = prostigmine bromide. Data in parentheses are for control eye.

preceding table, *i.e.*, neostigmine instilled into the eye caused in most cases definite inactivation of ChE in aq.h., as, for instance, in test 3 (Fig. 20, contractions 4—11), or complete inactivation, as in test 7 (Fig. 20, contractions 12—19). In two cases (tests 1 and 4), no definite change from the controls was demonstrable.

Regardless of whether the aq.h. was primary, secondary (tests 3, 4 and 5) or tertiary (test 6), the effect of neostigmine was similar in the respect that no increase, at least, occurred in the ChE activity.

After instillation of such large amounts of neostigmine that systemic symptoms were seen (in test 11 the rabbit began to tremble, in test 12 there was defecation and profuse salivation), no ChE activity was seen in the aq.h. of either eye. On the contrary, a suspicion of the possible presence of ACh in the aq.h. was entertained because the contractions of the leech back were very slightly larger than the control contractions (Fig. 21). Further evidence of a systemic effect by ACh was the slight ciliary congestion also seen in the untreated eye.

Two experiments were made to demonstrate the effect of parenteral administration of neostigmine on the ChE activity of aq.h. Subcutaneous injections were made of such large amounts of neostigmine that the animal in one experiment (test 13) showed distinct lassitude; in another experiment (test 14) profuse salivation, masseteric spasm, throat rattle, defecation and micturition were evidence of definite toxic action. The control aq.h. was drawn from one eye before injection and the test sample was taken from the other, intact eye after action of the injected substance. Mild ciliary congestion also in the unpunctured eye and distinct contraction of the pupils from their pre-injection size were again seen as signs of extension of the action to the eyes. — In all the neostigminised eyes of the rabbits there was definite and in most cases maximal miosis. — The results of the tests after subcutaneous injection indicate partial inactivation of ChE in aq.h. (test 13, Fig. 22, contractions 10 and 11) and, at the most, continued decrease of an originally weak or doubtful ChE activity (test 14). At least no increase in the ChE activity was demonstrable in any of the cases.

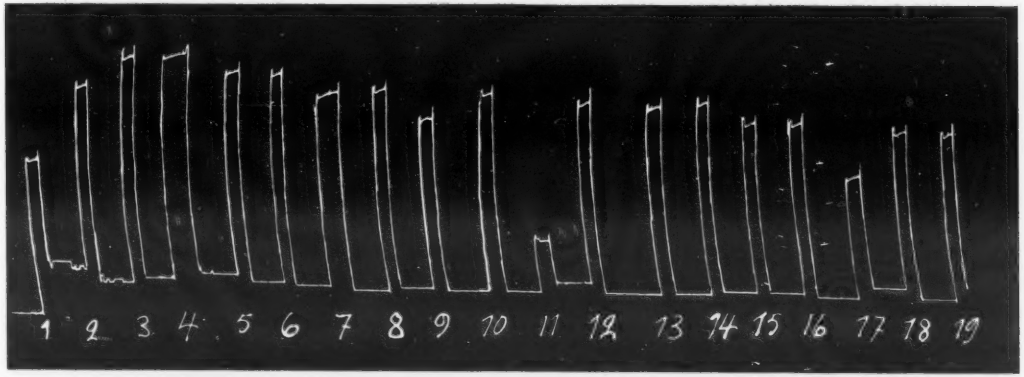
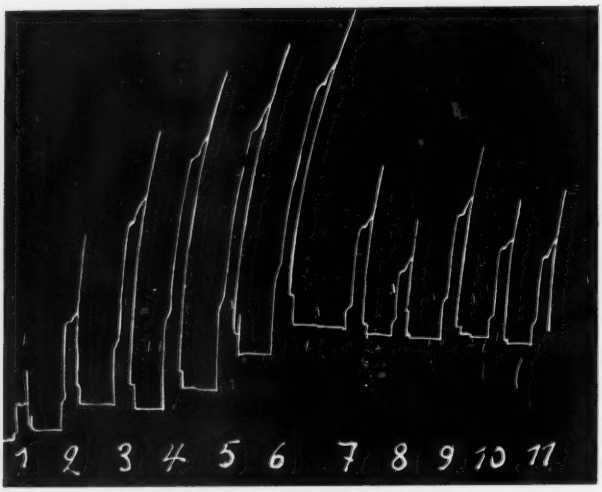
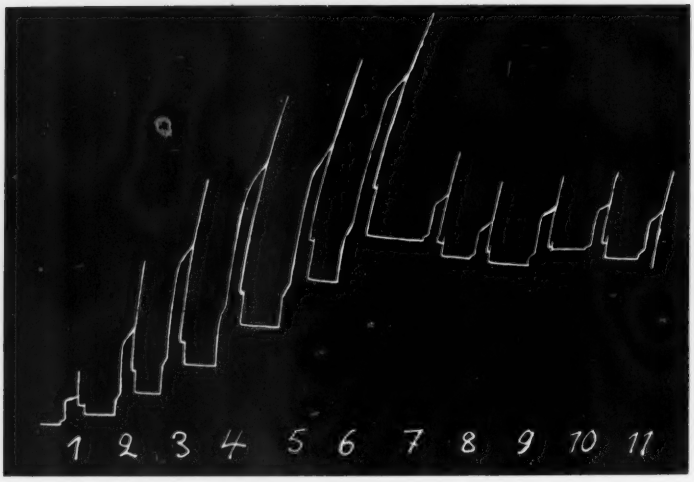


Fig. 20. — Effect of neostigmine on the ChE activity of the aq.h. 1, 2, 3, 4, 6, 8, 10, 12, 14, 16 and 18 = 0.5 μg ACh; 5 = 0.5 μg ACh + 0.1 cc secondary aq.h. from eye neostigminised after primary puncture; 9 = 5 incubated 1 hr.; 7 = 0.5 μg ACh + 0.1 cc secondary aq.h.; 11 = 7 incubated 1 hr. 13 = 0.5 μg ACh + 0.1 cc primary aq.h. of another rabbit; 17 = 13 incubated 1 hr.; 15 = 0.5 μg ACh + 0.1 cc primary aq.h. from neostigminised eye; 19 = 15 incubated 1 hr. Test object frog rectus.



a



b

Fig. 21. — Effect of neostigmine on the ChE activity of aq.h. a) 1 = 0.03 μg ACh; 2 and 5 = 0.05 μg ACh; 3 = 0.05 μg ACh + 0.05 cc aq.h. from neostigminised eye; 4 = 3 incubated ½ hr.; 6 = 3 incubated 1 ½ hrs.
b) 1 = 0.03 μg ACh; 2 and 5 = 0.05 μg ACh; 3 = 0.05 μg ACh + 0.05 cc aq.h. from control eye; 4 = 3 incubated ½ hr.; 6 = 3 incubated 1 ½ hrs.;

a) 7 = 0.02 μg ACh + 0.05 cc aq.h. from neostigminised eye of another rabbit; 8 and 10 = 0.02 μg ACh; 9 = 7 incubated 1 hr.; 11 = 0.22 μg ACh.
b) 7 = 0.02 μg ACh + aq.h. from control eye; 8, 10 and 11 = 0.02 μg ACh; 9 = 7 incubated 1 hr. Test object leech muscle.

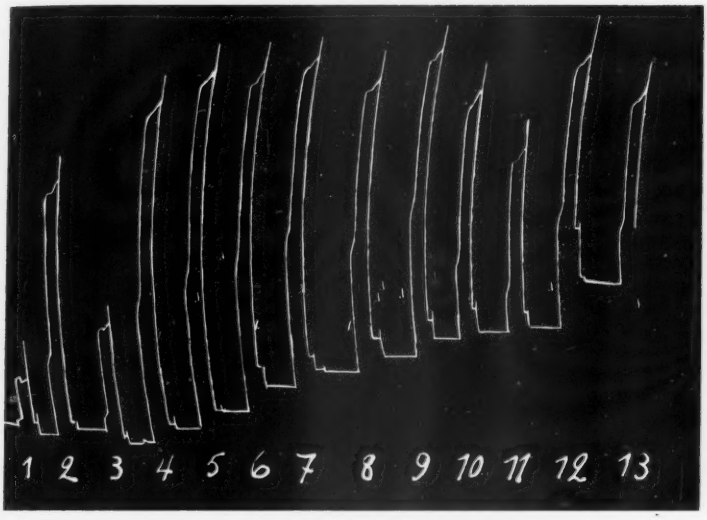


Fig. 22. — ChE activity in aq.h. of the rabbit after intravenous injection of neostigmine. 1 and 3 = 0.01 μg ACh; 2, 4, 9 and 12 = 0.02 μg ACh; 13 = 0.014 μg ACh; 5 = 0.02 μg ACh + 0.05 cc aq.h. from neostigmine-injected rabbit; 7 = 5 incubated 1 hr.; 10 = 5 incubated 2 ½ hrs.; 6 = 0.02 μg ACh + 0.05 cc aq.h. from control eye; 8 = 6 incubated 1 hr.; 11 = 6 incubated 2 ½ hrs. Test object leech muscle.

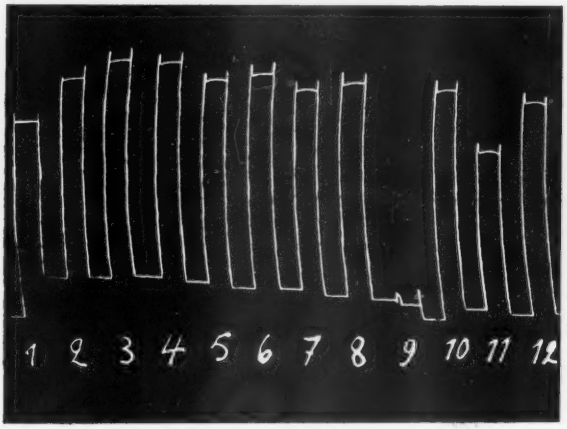


Fig. 23. — Effect of DFP on the ChE activity of aq.h. 1, 2, 3, 4, 6, 8, 10 and 12 = 0.5 μg ACh; 5 = 0.5 μg ACh + 0.05 cc aq.h. from DFP-instilled eye; 9 = 5 incubated 1 hr.; 7 = 0.5 μg ACh + 0.05 cc aq.h. from control eye; 11 = 7 incubated 1 hr. Test object frog rectus.

TABLE 14

EXPERIMENTS WITH DFP

Test No.	Concentr., per cent	No. of Drops	Time of Action, h. min.	Diam. of pupil, mm		Amt. of aq.h. per Puncture, cc		ChE in Prim. aq.h. (Control)	Effect on ChE in aq.h.			Aq.h. per Test, cc	Hydrolysis of ACh, μg			Test Object
				R.E.	L.E.	R.E.	L.E.		Inactivated	No Change	Increased		30 min.	1 h.	Over 1 h.	
1	0.1	2	1 50	2	4.5	0.19	0.25	+			+	0.05	0.5 [1]			Frog
														(0.1)		
2	»	1	2 15	3	7	0.16	0.25	+			+	»		0.5		»
		1	1											(0.1—0.2)	(0.3—0.4) [2]	
3	»	1	4 10	2	6.5	0.11	0.25	+			+	»		0.5		»
		2	1 45									(0.1)		(0.3)		
4	»	2	2 20	1	3.5	0.21	0.3	+			+	0.05	0.5			»
		2	55										(+)		(0.2) [3]	
5 [5]	»	2	4	4	6.5	0.13	?	+ [5]	—		+?	»		0.5		»
														(0.5)		·
6 [5]	»	2	3 30	6.5	6.5	0.28	—	[6]	—		+?	»	0.5			»
7	0.05	1	2 20	4	7.5	0.22	0.23	—		±	—	»	—		— [7]	Leech
8	0.1	1	2 10	2	6	0.23	0.31	+	+			»	—		— [4]	»
													(+)		(0.01–0.02) [4]	

[1] 15—30 min.
[2] 3 h. 30 min.
[3] 2 h.
[4] 1 h. 30 min.
[5] Secondary aq.h.
[6] No control.
[7] Graphs for treated and control eyes were identical. Test time 1 ½ h.

DFP was instilled into the right eye. Values in parentheses are for control eye. Amounts of ACh: frog muscle, 0.5 μg; leech back, 0.05 μg.

EXPERIMENTS WITH DFP

The DFP used was an 0.1 per cent solution in arachis oil (Boots Pure Drug Co., Ltd.). Using a normal dropper, 30 drops were obtained from 1 cc of solution, each drop thus containing 33.3 μg of DFP. However, it is difficult to evaluate the amount of DFP absorbed into the eye, since DFP is very poorly absorbed by the living cell. The absorbed amount is estimated to be only 0.5 per cent of the amount outside the cell (de Roetth 1951).

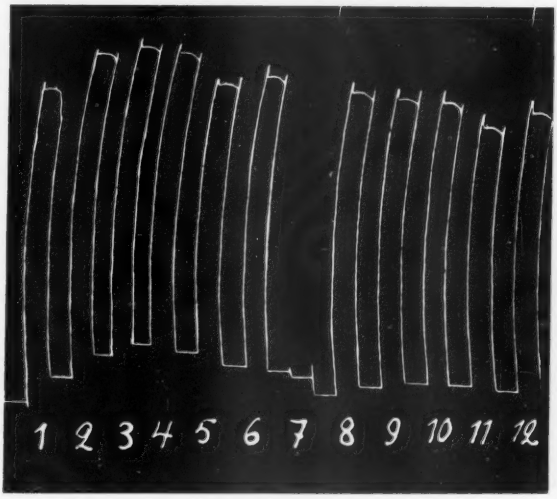


Fig. 24. — Effect of DFP on the ChE activity of aq.h. 1, 2, 3, 4, 6, 8, 10 and 12 = 0.5 μg ACh; 5 = 0.5 μg ACh + 0.05 cc aq.h. from DFP-instilled eye; 7 = 5 incubated ½ hr.; 9 = 0.5 μg ACh + 0.05 cc aq.h. from control eye; 11 = 9 incubated ½ hr. Test object frog rectus.

The 4 tests first listed in Table 14 show the marked increase in the ChE activity of aq.h. produced by instillation of DFP into the eye (Fig. 23, contractions 9 and 11; Fig. 24, contractions 7 and 11). The circumstance that the amount of aq.h. withdrawn from the DFP-instilled eye was in all cases unintentionally smaller than the control amount excludes the possibility of error through suction of ChE from the surrounding tissues by excessive aspiration. More exact quantitative determinations were not possible because of the few tests permitted by the inadequate amounts of aq.h.

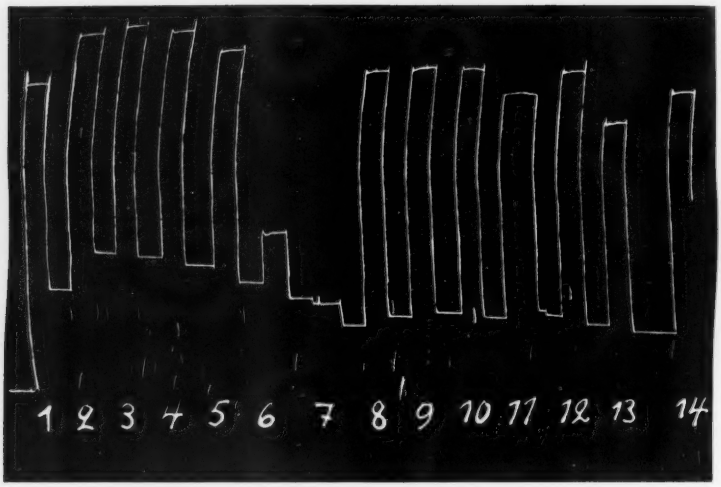


Fig. 25. — Effect of DFP on the ChE activity of aq.h. 1, 2, 3, 4, 8, 10 and 12 = 0.5 μg ACh; 14 = 0.45 μg ACh; 5 = 0.5 μg ACh + 0.05 cc aq.h. from DFP-instilled eye; 6 = 5 incubated 15 min.; 7 = 5 incubated ½ hr.; 9 = 0.5 μg ACh + 0.05 cc aq.h. from control eye; 11 = 9 incubated ½ hr.; 13 = 9 incubated 1 hr. Test object frog rectus.

and the slow reaction of the test objects. The graph in Fig. 25 (test 1), however, is evidence that the ChE activity in the aq.h. in this test was sufficiently great to hydrolyse 0.5 μg of ACh in 15—30 min.

The uneven basic line seen in several of the graphs, as for instance in Fig. 25, was not always avoidable since the amounts of ACh varied greatly. However, it has very little bearing on the evaluation of the results, since the standard control is an invariable criterion.

Tests 5 and 6 were carried out with secondary aq.h. withdrawn 4 hrs. 10 min. and 4 hrs. 30 min. after the primary puncture. In these tests the ChE activity of the aq.h. of the DFP-instilled eye was so great that 0.05 cc of aq.h. hydrolysed 0.5 μg of ACh in 1 hr. (test 5, Fig. 26, contractions 3 and 7) and in 30 min. (test 6). Since the ChE activity of secondary aq.h. from the animal's untreated eye was also capable of hydrolysing a corresponding amount of ACh (Fig. 26, contractions 5 and 9), it is not possible to conclude from these tests, in the absence of control contractions, which eye had the higher ChE activity, but no ChE inhibition, at least, could be observed.

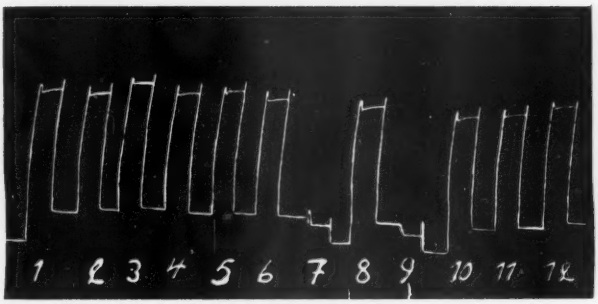
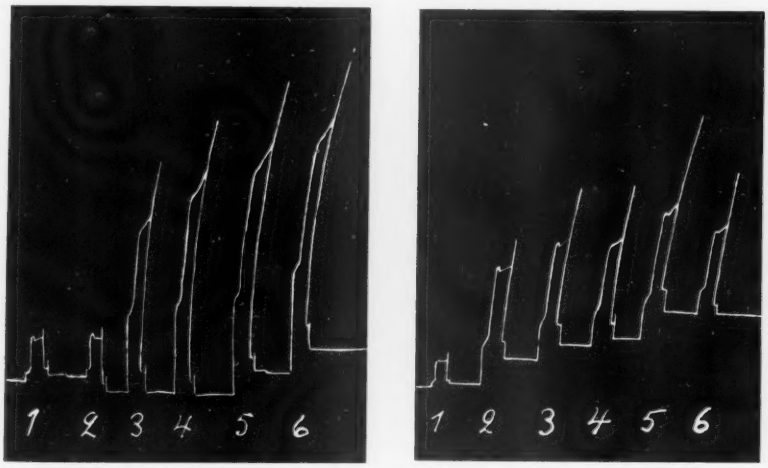


Fig. 26. — Effect of DFP on the ChE activity of secondary aq.h. 1, 4, 6, 8, 10 and 12 = 0.5 μg ACh; 2 = 0.5 μg ACh after 1 hr. 15 min. in thermostat at +37° C; 11 = 0.5 μg ACh after 3 ½ hrs. at +37° C; 3 = 0.5 μg ACh + 0.05 cc sec. aq.h. from eye DFP-instilled after primary puncture; 7 = 3 incubated 1 hr.; 5 = 0.5 μg ACh + 0.05 cc sec. aq.h.; 9 = 5 incubated 1 hr. Test object frog rectus.

An opposite effect of DFP is seen in tests 7 and 8 (Figs. 27 a and b). Complete inactivation of ChE in aq.h. occurred in test 8; the control, it is true, also had a low ChE content. This was probably a rabbit with individually low ChE values. It is to be



a b

Fig. 27. — Effect of DFP on the ChE activity of aq.h. a) 1 = 0.03 μg ACh; 2 = 0.02 μg ACh; 5=0.05 μg ACh; 3 = 0.05 μg ACh + 0.05 cc aq.h. from DFP-instilled eye; 4 = 3 incubated ½ hr.; 6 = 3 incubated 1 ½ hrs.

b) 1 = 0.02 μg ACh; 2 and 5 = 0.05 μg ACh; 3 = 0.05 μg ACh + 0.05 cc aq.h. from control eye; 4 = 3 incubated ½ hr.; 6 = 3 incubated 1 ½ hrs. Test object leech muscle.

noted, furthermore, that the DFP concentration in test 7 was lower than in the other tests and the miosis was not of maximum degree.

The tests with DFP definitely indicate that instillation of an 0.1 per cent solution of DFP into the eye frequently increases the ChE activity of the aq.h.

EXPERIMENTS WITH TEPP

The vehicle used for TEPP, as well as for HETP and Tabun, was anhydrous propylene glycol (The British Drug Houses, Ltd.) which, as an additional precaution, was distilled twice before use.

In these experiments, the aq.h. samples from the treated and control eyes were tested consecutively on the same muscle or parallel on a pair of muscles. Experience has shown that similarly

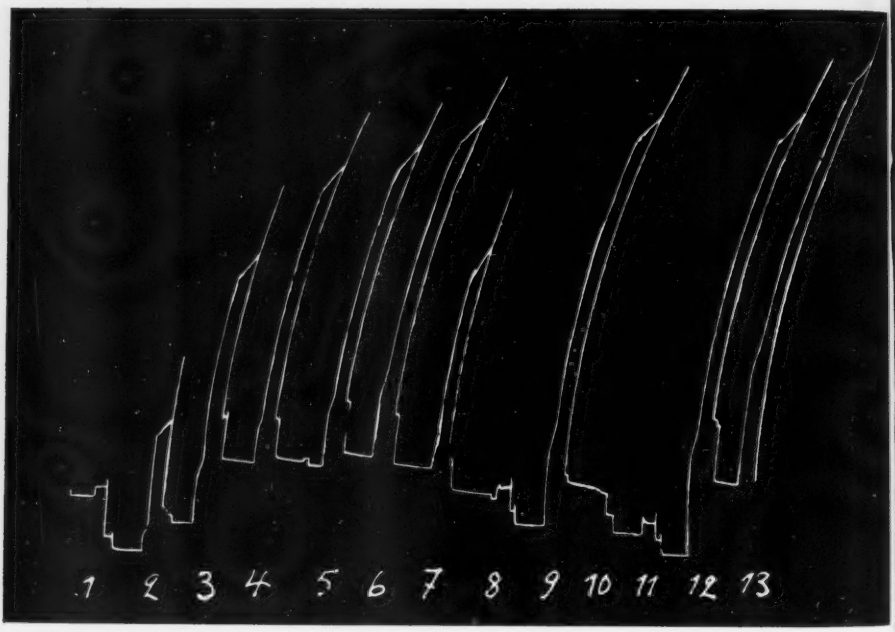


Fig. 28. — Effect of TEPP on the ChE activity of aq.h. 1 = 0.03 μg ACh; 2, 3, 6, 9 and 13 = 0.05 μg ACh; 4 = 0.05 μg ACh + 0.05 cc aq.h. from control eye; 7 = 4 incubated 1 ½ hrs.; 5 = 0.05 μg ACh + 0.05 cc aq.h. from TEPP-instilled eye; 8 = 5 incubated 1 ½ hrs.; 10 = no significance; 11 = 0.01 μg ACh; 12 = 0.04 μg ACh. Test object leech muscle.

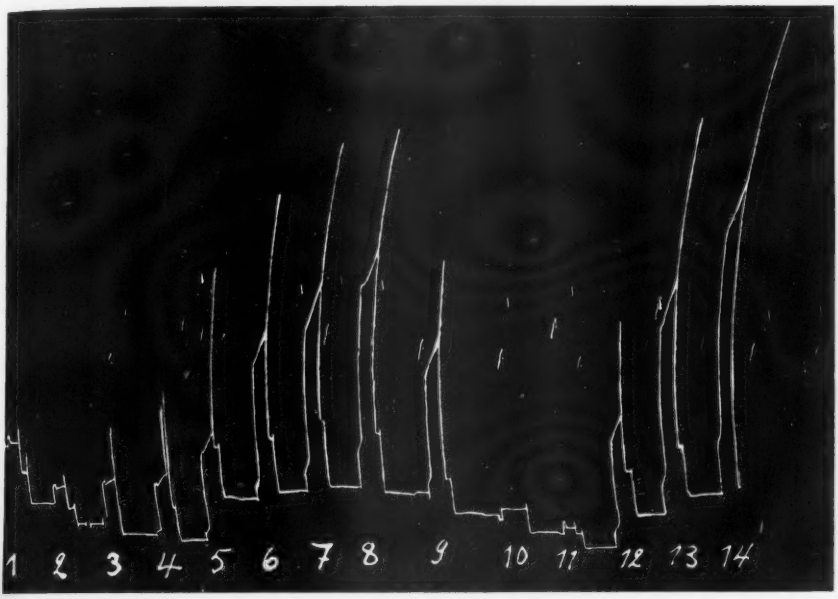


Fig. 29. — Effect of TEPP on the ChE activity of aq.h. 1 = 0.03 μg ACh; 2, 3, 4, 5, 8 and 14 = 0.05 μg ACh; 6 = 0.05 μg ACh + 0.05 cc aq.h. from control eye; 9 = 6 incubated 1 ½ hrs.; 7 = 0.05 μg ACh + 0.05 cc aq.h. from TEPP-instilled eye; 10 = 7 incubated 1 ½ hrs.; 11 = 0.01 μg ACh; 12 = 0.03 μg ACh; 13 = 0.04 μg ACh. Test object leech muscle.

treated muscle pairs — especially the length of relaxation time before tests is important — give very similar, and sometimes even identical, reactions, greatly facilitating comparison of the results. A further advantage is the possibility to perform the tests and controls simultaneously in every respect, to store the solutions and samples identically, etc. When the tests are made with the same muscle, the contractions are directly comparable.

Fig. 28 is the curve for test 4, in which the control was tested on the same leech muscle piece as the actual sample. It will be seen that TEPP definitely increased the ChE activity of the aq.h., since the ACh was hydrolysed to nearly 0 in 1½ hrs., whereas the control still produced a marked contraction (contractions 8 and 7). The same curve shows also comparable quantitative contractions, in which 0.01 μg of ACh is still clearly demonstrable (contraction 11), the muscle having retained its sensitivity to differences of, at least, 0.01 μg of ACh (contractions 12 and 13, corresponding to 0.04 and

0.05 μg). In this curve, also, the contractions produced by small amounts of ACh relax rapidly, causing an uneven basic line, as was mentioned earlier.

A result nearly identical with the above is seen in Fig. 29, test 5, in which the aq.h. samples of another rabbit were tested on the other back muscle of the same leech. Hydrolysis of ACh in 1½ hrs. (contractions 10 and 9) was quantitatively of the same order as in the preceding test. Contractions 11—14 at the end of the curve are quantitative ACh controls.

Table 15 combines the data on the tests with TEPP. It is seen that the effect of TEPP on the ChE activity of aq.h. varied according to the concentration of the test substance. While low concentrations of 0.1 and 1.0 (sometimes 2.0) per cent inhibited the action of ChE, a 2.0 per cent solution appeared to have usually a definitely increasing effect on the activity.

TABLE 15
EXPERIMENTS WITH TEPP AND HETP

EXPERIMENTS WITH HETP

The results of the tests with HETP are also included in Table 15. Figs. 30 a and b show the curves for test 1. The leech used in this test had a comparatively low sensitivity and there are no very conspicuous differences between the contractions; however, they are sufficiently distinct to permit evaluation of the results. The slightly higher columns obtained for ACh controls 3 and 5, in comparison to the initial contraction 1, is a prevalent occurrence in all the tests with leech back, since the sensitivity of this muscle to ACh increased almost constantly during each test. It will be observed from the curves that 0.05 cc of aq.h. from the control eye caused a small degree of ACh hydrolysis already in 30 min., and that *c.* 0.01 μg/0.05 μg was hydrolysed in 1½ hrs., as estimated by means of the quantitative control (contraction 6). The sample from the same rabbit's HETP-treated eye, tested on the other back muscle of the same leech, remained definitely on a level with the ACh control for 1½ hrs. Contractions 6 and 7 proved that the muscle was still sufficiently sensitive to register quantitative differences with an accuracy of at least 0.01 μg, regardless of the small amplitude of its contractions. These tests may therefore be regarded as evidence of the inactivation of ChE in aq.h. by HETP.

TABLE 15

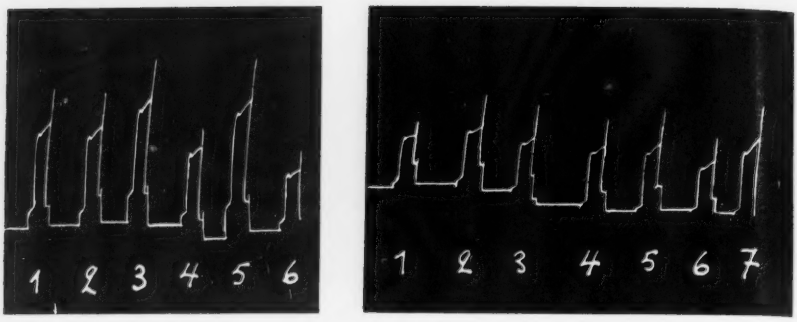
EXPERIMENTS WITH TEPP AND HETP

Test No.	Test Substance	Concentr., per cent	No. of Drops	Time of Action, h. min.	Amt. of aq.h. per Puncture, cc		ChE in Prim. aq.h. (Control)	Effect on ChE in aq.h.			Aq.h. per Test, cc	Hydrolysis of ACh, μg				
					R.E.	L.E.		Inactivated	No Change	Increased		30 min.	1 h.	1 h. 30 min.	2 h.	3 h. or more
1	TEPP	0.1	1	1			+	+			0.05	—		—		
		1.0	1	1 15								(+?)		(0.02)		
2	»	0.1	1	1			—		±		»		—		—	
													(—)		(—)	
3	»	2.0	1	2 45			+	+			»		—			+? [1]
													(+?)			(0.01) [1]
4	»	»	1	2			+			+	»			0.04		
														(0.02)		
5	»	»	1	3 40			+			+	»			0.04		
														(0.01—0.02)		
6	»	»	1	4 45			+			+	»		0.03			0.05 [2]
													(0.02)			(0.03—0.04) [2]
1	HETP	0.25	1	1 40	0.16	0.21	+	+			»	—		—		
												(+)		(0.01)		
2	»	»	1	2	0.2	0.23	+	+			»	+		0.02		
												(0.02)		(0.05)		
3	»	»	1	1 45	0.21	0.22	+			+	»	+		0.03	0.04	
												(+)		(0.02)	(0.03)	
4	»	0.5	1	1 30	0.22	0.3	+				»	+		0.01		
									±			(+)		(0.01)		
5	»	»	1	1 55	0.2	0.32	—			+	»	0.05				
												(—)		(—)		
6	»	1.0	1	2 30	0.26	0.28	—			+	»	+		0.03—0.04		
												(—)		(—)		
7	»	»	1	4 15	0.25	0.31	+?			+	»	—?		0.02—0.03		
												(+?)		(+?)		

[1] 4 h.

[2] 2 h. 30 min.

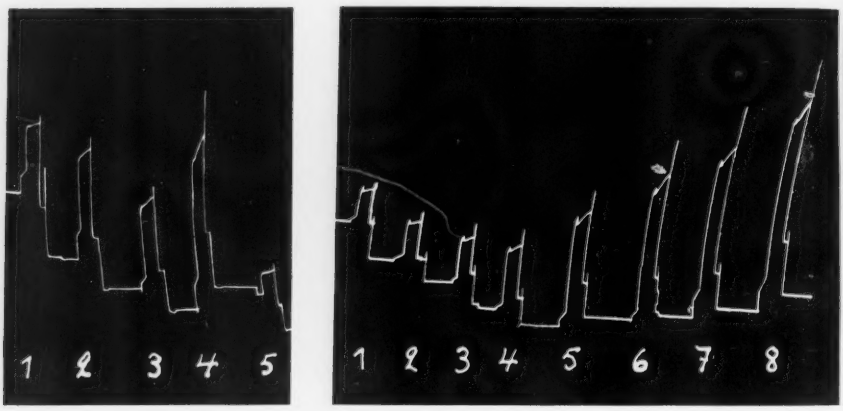
Test substance was instilled into the right eye. Values in parentheses are for control eye. Test object leech back.



a b

Fig. 30. — Effect of HETP on the ChE activity of aq.h. a) 1 = 0.05 μg ACh + 0.05 cc aq.h. from control eye; 2 = 1 incubated ½ hr.; 4 = 1 incubated 1 ½ hrs.; 3 and 5 = 0.05 μg ACh; 6 = 0.03 μg ACh.

b) 1 = 0.05 μg ACh + 0.05 cc aq.h. from HETP-instilled eye; 2 = 1 incubated ½ hr.; 4 = 1 incubated 1 ½ hrs.; 3, 5 and 7 = 0.05 μg ACh; 6 = 0.04 μg ACh. Test object leech muscle.



a b

Fig. 31. — Effect of HETP on the ChE activity of aq.h. a) 1 and 4 = 0.05 μg ACh; 2 = 0.05 μg ACh + 0.05 cc aq.h. from HETP-instilled eye; 3 = 2 incubated ½ hr.; 5 = 2 incubated 1 ½ hrs.

b) 1, 2, 3, 4 and 7 = 0.05 μg ACh; 5 = 0.05 μg ACh + 0.05 cc aq.h. from control eye; 6 = 5 incubated ½ hr.; 8 = 5 incubated 1 ½ hrs. Test object leech muscle.

The curves for test 6 are shown in Figs. 31 a and b. According to the first curve (a), aq.h. from the HETP-treated eye was clearly capable of hydrolysing already in 30 min. a portion of the ACh (contraction 3) and in 1½ hrs. nearly the entire amount, which

was estimated at 0.03—0.04 μg/0.05 μg (contraction 5). The corresponding control (curve b) does not indicate any demonstrable ChE activity in the aq.h. (contraction 8). In contradistinction to the results obtained in test 1, these results are definite evidence that HETP caused an increase in the ChE activity of aq.h.

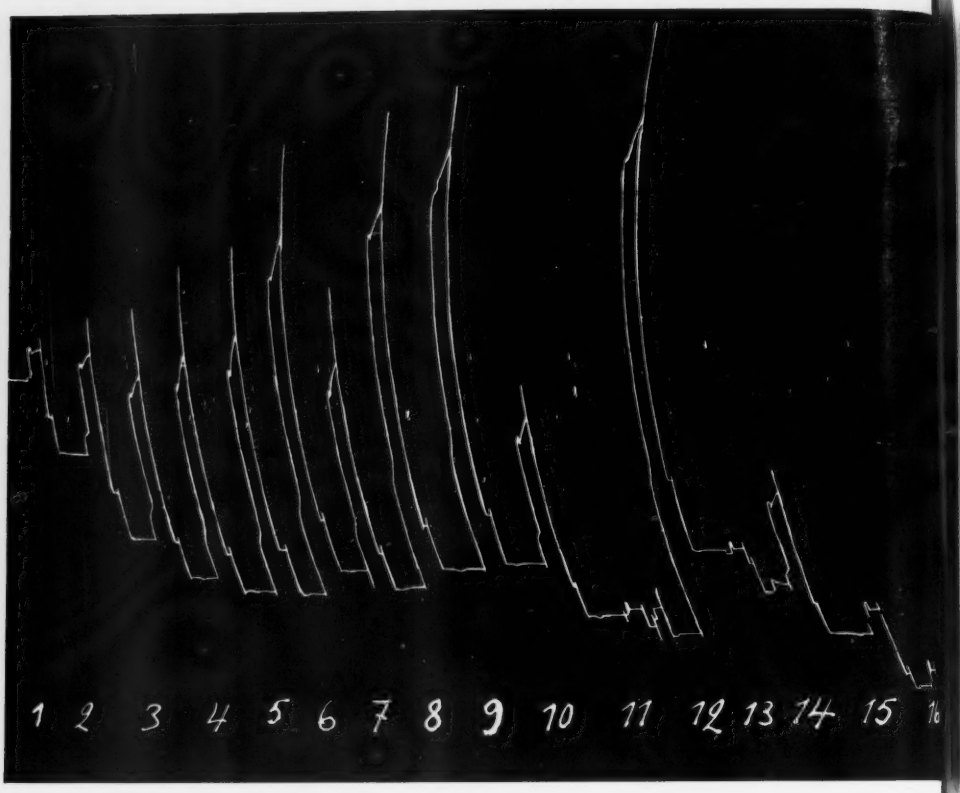
The test results listed in Table 15 are greatly similar to those obtained with TEPP, *i.e.*, the low concentrations of HETP caused inactivation of the ChE, whereas higher concentrations increased the activity. Comparison of the concentrations indicates that HETP produced increased ChE activity in comparatively lower concentrations than TEPP. However, in comparison to the tests with DFP, for instance, the effective HETP and TEPP concentrations were of the same order of magnitude and higher than that of DFP.

EXPERIMENTS WITH MINTACOL

The tests with Mintacol were carried out with one concentration of 1: 6,000 (0.017 per cent solution), since this concentration has been taken into use by the manufacturers (Bayer, Leverkusen). It was considered interesting to observe the effect of this concentration on the ChE activity of aq.h. The test results are seen in Table 16.

The first part of the curves in Figs. 32 a and b refer to test 3 and the latter part to test 4. In test 3 it is not possible to observe any difference in the ChE activities of aq.h. from the Mintacol-treated and the control eye, for the shape of the curves is identical. However, it definitely indicates the presence of ChE in the aq.h. of both eyes. Test 4, again, covers a case in which the aq.h. from the control eye (contractions 9—11) showed approximately the same ChE activity as in test 3 but the aq.h. from the Mintacol-treated eye evidenced considerably greater activity. An amount of 0.07 cc of the latter aq.h. almost totally hydrolysed 0.05 μg — or at the minimum 0.04 μg — of ACh already in 1 hr. The quantitative controls are seen in the last section of the curves.

The most interesting of the tests with Mintacol is probably test 1 (Figs. 33 a and b), which brings out the effect of Mintacol and physostigmine on the aq.h. of the same rabbit. Two drops of 0.017 per cent Mintacol were allowed to act for 55 min. on the animal's right eye, at the same time as 2 drops of 0.5 per cent

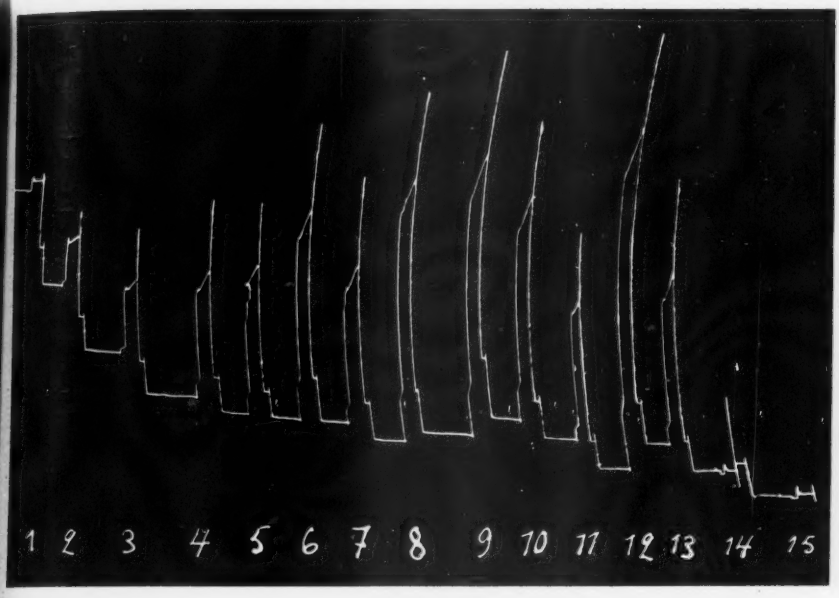


a

Fig. 32. — Two tests for the effect of Mintacol on the ChE activity of aq.h. a) 1 = 0.03 µg ACh; 2, 3, 6, 8 and 12 = 0.05 µg ACh; 4 = 0.05 µg ACh + 0.05 cc aq.h. from control eye; 5 = 4 incubated ½ hr.; 7 = 4 incubated 1 ½ hrs. b) 1 = 0.03 µg ACh; 2, 3, 6, 8 and 12 = 0.05 µg ACh; 4 = 0.05 µg ACh + 0.05 cc aq.h. from Mintacol-instilled eye; 5 = 4 incubated ½ hr.; 7 = 4 incubated 1 ½ hrs.

a) 9 = 0.05 µg ACh + 0.07 cc aq.h. from Mintacol-instilled eye of another rabbit; 10 = 9 incubated ½ hr.; 11 = 9 incubated 1 hr.; 13 = 0.01 µg ACh; 14 = 0.02 µg ACh; 16 = 0.01 µg ACh; 15 = b) 4 incubated 5 hrs. 10 min. b) 9 = 0.05 µg ACh + 0.07 cc aq.h. from control eye; 10 = 9 incubated ½ hr.; 11 = 9 incubated 1 hr.; 13 = 0.03 µg ACh; 15 = 0.01 µg ACh; 14 = a) 4 incubated 5 hrs. Test object leech muscle.

physostigmine acted on the left eye. The test showed no ChE activity in the aq.h. of the physostigminised eye (Fig. 33 b, contractions 2, 3 and 5). It is possible that there was initially no ChE in the aq.h. or that a small normal amount was completely inactivated.



b

The aq.h. from the eye instilled with Mintacol, on the other hand, showed such marked ChE activity that nearly the total amount of (0.05 μg) ACh was hydrolysed in 30 min. (Fig. 33 a, contraction 3) and the total amount (0.04—0.05 μg/0.05 μg) in 1½ hrs. (contraction 5). Since in the light of the other tests the normal ChE activity was not so high, it seems probable that considerable increase in ChE activity had occurred. This test thus brings out most clearly the divergent action of these two different types of AntiChE:s on the ChE activity of aq.h.

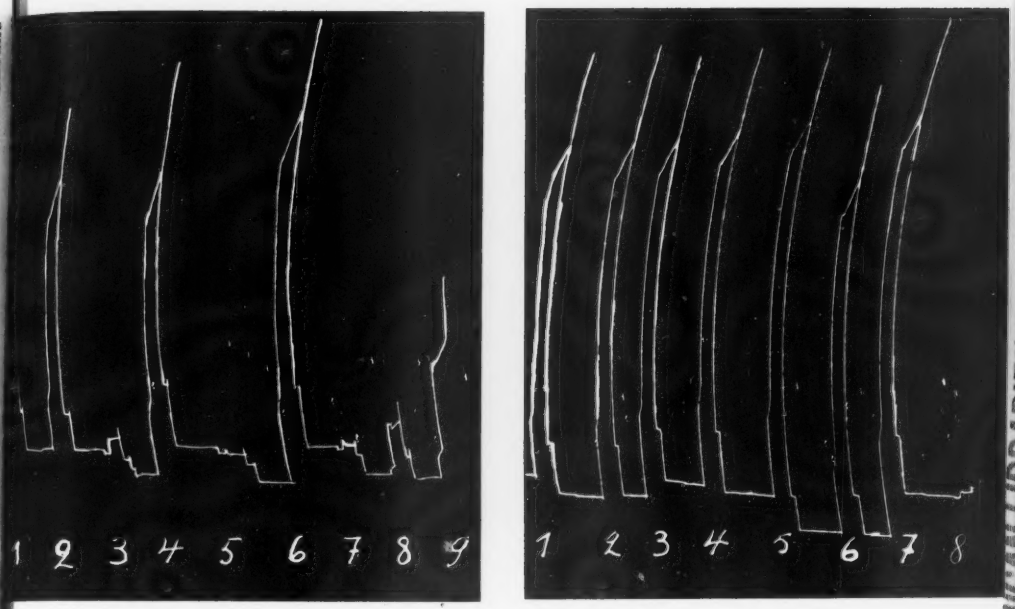
The test results listed in Table 16 vary between increase and inhibition of ChE activity. On the basis of these tests and the preceding series of tests with other AntiChE:s, it seems probable that *inactivation of ChE commonly occurs with decreasing concentrations of Mintacol, while the activity is enhanced by increasing concentrations.*

TABLE 16

EXPERIMENTS WITH MINTACOL AND TABUN

Test No.	Test Substance	Concentr., per cent	No. of Drops	Time of Action, h. min.	Amt. of aq.h. per Puncture, cc		ChE in Prim. aq.h. (Control)	Effect on ChE in aq.h.			Aq.h. per Test, cc	Hydrolysis of ACh, μg				
					R.E.	L.E.		Inactivated	No Change	Increased		30 min.	1 h.	1 h. 30 min.	2 h.	Over 2 h.
1	Mint.	0.017	2	55	0.2	0.15	[1]			+	0.045	0.03—0.04 (—?)		0.04—0.05 —		
2[2]	»	»	2	2	0.23	0.23	+[2]			+?	0.65	(0.02)		0.02—0.03 (0.04)		
3	»	»	2	1 10	0.23	0.25	+		±		0.05	+ (+)		0.02 (0.02)		
4	»	»	2	1 30	0.25	0.25	+			+	0.07	0.02 (0.01)	0.04 (0.02)			
5	»	»	2	2	0.2	0.25	+			+	0.06		0.05 (0.03)			
6	»	»	4	2 30	0.11	0.25	[3]			+(?)	0.05	0.03			+	
1	Tabun	0.005	1	1 50	0.19	0.20	+	+			0.05		0.01 (0.02)	0.02 (0.05)		
2	»	»	1	2 15	0.23	0.24	±		±		»	+ (+)		0.005[7] (»)		
3	»	»	1	3 45	0.24	0.26	+		±		»	+ (+?)		0.005[7] (+)		(0.01)[4]
4	»	»	1	3 45	0.21	0.25	+			+	»	0.05 (+?)		0.01)		
5	»	»	2	1 50	0.17	0.2	—			+			+ (—)			0.02—0.03[5] (—)[5]
6	»	0.05	1	1 50	0.2	0.27	+			+	»	0.05 (—)			(0.01)	
7	»	»	1	1 45	0.21	0.28	+			+	»	0.05 (+)				(0.03)[6]
8	»	»	1	2	0.2	0.22	—			+	»		0.05 (—)		(—)	

[1] Not tested. [2] Secondary aq.h. [3] Primary aq.h. was not available, as eye had been treated with neostigmine. The neostigmine-instilled eye showed slight ChE activity. [4] 2 h. 30 min. [5] 3 h. 20 min. [6] 3 h. 30 min. [7] ACh amount 0.02 μg. in other tests 0.05 μg.



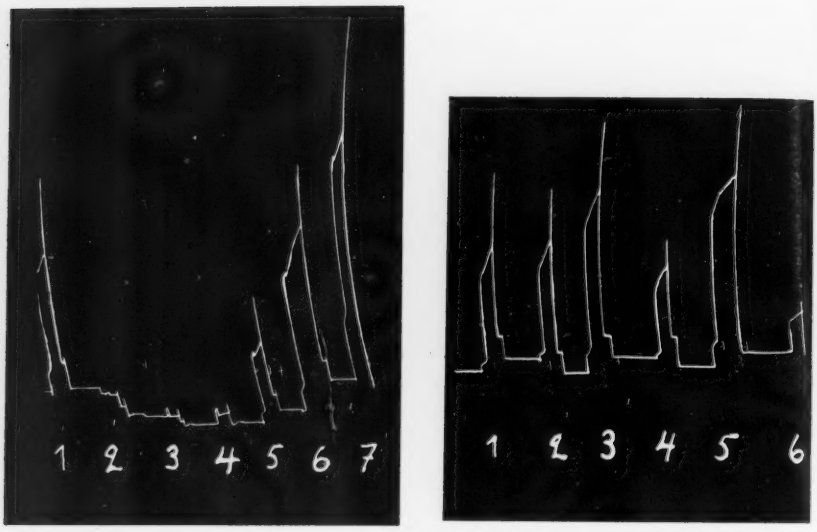
a b

Fig. 33. — Test for the ChE activity of aq.h. after instillation of Mintacol into one eye and of physostigmine into the other eye of the same rabbit. a) 1, 4 and 6 = 0.05 μg ACh; 7 = 0.01 μg ACh; 8 = 0.02 μg ACh; 9 = 0.03 μg ACh; 2 = 0.05 μg ACh + 0.045 cc aq.h. from Mintacol-instilled eye; 3 = 2 incubated ½ hr.; 5 = 2 incubated 1½ hrs.

b) 1, 4 and 7 = 0.05 μg ACh; 6 = 0.04 μg ACh; 8 = 0.01 μg ACh; 2 = 0.05 μg ACh + 0.045 cc aq.h. from physostigminised eye; 3 = 2 incubated ½ hr.; 5 = 2 incubated 1½ hrs. Test object leech muscle.

EXPERIMENTS WITH TABUN

Table 16 also includes the results of the tests with Tabun. These results are very distinct. Instillation of 0.005 per cent Tabun into the eye caused contraction of the pupil but not the maximum degree of miosis. The effect on the ChE activity of aq.h. was either insignificant, slightly inactivating, or slightly enhancing. A concentration of 0.05 per cent, on the other hand, produced rapidly (in 7—15 min.) maximum miosis, definite ciliary congestion and a greatly increased ChE activity. A case of this kind is test 4 (Figs. 34 a and b). The control eye (curve b) manifested normal, clearly demonstrable ChE activity, which hydrolysed 0.01 μg/0.05 μg of



a b

Fig. 34. — Effect of Tabun on the ChE activity of aq.h. a) 1 = 0.05 μg ACh + 0.05 cc aq.h. from Tabun-instilled eye; 2 = 1 incubated ½ hr.; 3 = 0.01 μg ACh; 4 = 0.02 μg ACh; 5 = 0.03 μg ACh; 6 = 0.04 μg ACh; 7 = 0.05 μg ACh.

b) 1 = 0.05 μg ACh + 0.05 cc aq.h. from control eye; 2 = 1 incubated ½ hr.; 3 and 5 = 0.05 μg ACh; 4 = 1 incubated 1 ½ hrs.; 6 = 0.03 μg ACh. Test object leech muscle.

ACh in 1½ hrs. (contractions 1, 2 and 4), while the aq.h. from the Tabun-treated eye (0.05 cc) hydrolysed the total amount of 0.05 μg of ACh in already 30 min. (curve a, contractions 1 and 2).

EVALUATION OF THE RESULTS

It is to be pointed out that the results obtained with AntiChE:s in Part II of this investigation are not fully comparable in view of the variable concentrations and amounts of the instilled substances. These variations were employed for the purpose of studying the clinically used concentrations of those AntiChE:s that already have an established use. With the newer substances which are still in the experimental stage, the effort was to find the concentrations that had an evident effect on the test animal when miosis was used as the criterion. Thus, for instance, when TEPP in a 0.1 per cent concentration proved incapable of producing miosis, the 10 times

stronger concentration (1.0 per cent) was used. Grant (1948), it is true, usually employed 0.1 per cent TEPP.

It is a conspicuous feature of the results that the experiments with physostigmine and neostigmine showed no increase of ChE activity in the aq.h., even in cases in which the experimental animal had definitely toxic symptoms because of the large amount of test substance administered. On the other hand, the tests with DFP, TEPP, HETP, Mintacol and Tabun gave results that indicated in all these groups, and already after therapeutic doses, a more or less increased ChE activity of the aq.h.

Cases in which no definite difference was seen in relation to the control aq.h. (±) were present not only in the physostigmine and neostigmine groups but also in the polyalkylphosphate group. The majority of these results are ascribable to the inadequate sensitivity of the method.

The polyalkylphosphate group also showed some inactivation of ChE in aq.h. However, this occurred usually with low concentrations of the test substance. It appears probable that increasing ChE activity of the aq.h. is seen with rising concentrations of instilled polyalkylphosphates.

When inactivation of ChE occurred, the curves rarely showed marked differences from the controls in the amplitude of contractions which frequently was the case in the curves for increased ChE activity. It is obvious, however, that the differences cannot be very significant, for also the ChE activity of normal aq.h. is manifested only as small differences in ACh hydrolysis. In certain sample tests, however, the inactivation of ChE by physostigmine and neostigmine was so powerful that the inhibition of the increased ChE activity in the secondary aq.h. could also be demonstrated. In the experiments on the effect of DFP on ChE activity in secondary aq.h., no marked differences were seen from the controls, but they indicated at least that no inactivation of ChE occurred, contrary to the finding in the experiments with physostigmine.

In most cases miosis was of maximum degree and was thus a constant quantity. Variations in the amount of aq.h. withdrawn by puncture likewise had no decisive effect.

The action time allowed in the experiments varied from 55 min. to 4 hrs. 45 min. It appears probable that prolongation of the time of action within these limits enhances the main trend of

effect in each group, *i.e.*, promotes the inactivation of ChE in aq.h. by physostigmine and neostigmine and increases the tendency of polyalkylphosphates to augment ChE activity. However, the length of action is probably able to influence the effect of the substances to a very limited degree only. This may be inferred from a case of glaucoma studied by Grant (1948), in which TEPP increased the intraocular tension. Already after 24 hrs. the aq.h. was normal, *i.e.*, clear (the report gives no data on the protein content of the aq.h.). The results obtained in the present experiments (page 53) on the tendency of tertiary aq.h. to revert to normal justify this conclusion of a gradual disappearance of the effect on ChE activity when the action of the drugs subsides.

In Part II the same observation was made as in Part I (page 39) that the hydrolysing action of ChE on ACh, expressed in percentages, is similar regardless of 10-fold differences in the substrate (ACh), when tested on frog rectus and leech back. However, this cannot lead to erroneous interpretations of the relative effects of AntiChE:s, since the control was always carried out under the same test conditions.

The somewhat more frequent negative findings of ChE activity in primary aq.h. in the experiments with polyalkylphosphates than in the experiments in Part I create a suspicion of a slight systemic effect which extends to the other eye and produces inhibition of ChE in the control eye.

If a classification of the polyalkylphosphates according to the strength of their effect is justified on the basis of the poorly comparable experimental results obtained in the present investigation, the author is inclined to consider Tabun, Mintacol and DFP approximately equal in potency and definitely more powerful than TEPP and HETP. The latter two substances appear to be equal in effect; however, TEPP is probably slightly weaker than HETP.

DISCUSSION

An observation frequently made in earlier investigations appeared probable also in the investigation here described, *i.e.*, that especially the new AntiChE:s — polyalkylphosphates — increase vascular permeability and cause the formation of plasmoid aqueous. It seems surprising that they usually do not simultaneously inactivate the increased ChE activity of the aq.h. but, on the contrary, remarkably often augment it, whereas the reversible AntiChE:s — physostigmine and neostigmine — exert a definitely inactivating action on the ChE of aq.h. This seems to explain why von Sallmann failed to find any increase in ACh concentration in aq.h. after instillation of DFP (page 83).

Certain essential differences in the action of the above mentioned reversible AntiChE:s and polyalkylphosphates are already known. Thus polyalkylphosphates are capable of inactivating considerably more readily the non-specific than the specific ChE. Another significant difference is the protection of ChE against the action of polyalkylphosphates by physostigmine and neostigmine, among other substances, but not *vice versa*. In the light of the present investigation it appears probable that the effects of these substances on the eye also follow the same group distribution, since polyalkylphosphates frequently increased the ChE activity of aq.h., whereas similar action by physostigmine and neostigmine could not be demonstrated.

Why do polyalkylphosphates, regardless of the AntiChE properties which they possess, increase the ChE activity of aq.h.? It may merely be due to the diffusion into the a.ch. — because of the increased capillary permeability — of such a large amount of ChE that the instilled amount of AntiChE is not sufficient to inactivate it, especially in view of the poor absorption of AntiChE:s

into the living cell. The capillary injury produced by physostigmine and neostigmine would thus be considerably smaller.

On the other hand, the action of physostigmine on the cornea may provide an explanation for the amount of substances absorbed into the a.ch. This drug has been found to increase the action of certain anaesthetics on the cornea. It might be presumed that physostigmine at the same time renders the cornea more permeable to itself, enabling it to reach the a.ch. in a less diluted condition. There are no reports in the literature of similar effects by polyalkylphosphates.

A more complex explanation may also be found for the increase in ChE activity. If we proceed from the basis that the ChE of aq.h. originates in plasma, which in the rabbit contains chiefly specific ChE, as claimed by Mendel, Mundell and Rudney (1943) and Hawkins and Mendel (1947), the ChE entering the a.ch. of the rabbit would also be mainly specific in type. In view of the selective affinity of AntiChE:s for specific and non-specific ChE it would then seem logical that physostigmine and neostigmine would be able to inhibit the activity of the specific ChE in this aq.h., whereas polyalkylphosphates, with the exception of Tabun, would be practically ineffective. Ellis (1947) also observed that the serum of some rabbits is entirely incapable of hydrolysing BCh. The above hypothesis, however, is contradicted by the studies of Denys and Lévy (1948), according to which rabbit plasma contains more non-specific than specific ChE. The author's experiments with ChE of secondary rabbit aq.h. also seem to indicate that it is of the non-specific type.

However, since contraction of the pupil is obtained and maintained with AntiChE substances of these two groups, the ChE activity of aq.h. seems to play no part in the contraction. The miosis is evidently determined by the iris, the ChE content of which, according to the studies of de Roetth (1950), is decreased after instillation of DFP. This supposition is in harmony with the theory suggested by Dale (1948) that the effect of substances is different inside and outside a cell.

Dunphy (1949) pointed out that signs of congestion produced, for instance, by DFP appear only 10—24 hrs. after administration. In the rabbit experiments in the present investigation, however, ciliary congestion was usually seen already within the first few

hours and the increased ChE activity of the aq.h. indicated that the formation of plasmoid aqueous had started. In fact, Dunphy stated that it is difficult to explain the delayed vasodilatory symptoms.

The attention of several investigators has been drawn to the circumstance that, at the same time as these substances have proved highly advantageous by lowering the intraocular tension in aphakic glaucoma, they have been free of the undesirable effects of ciliary spasm and congestion. Leopold and Comroe (1946 a) suggested a theoretic explanation for this in the broken fibres of Zinn, which is the anatomic difference between these and normal eyes. However, this may probably account for the absence of pain due to spasm of accommodation, but not for the absence of congestive symptoms. Dunphy (1949) proposed in this connection that part of the rich ChE content of the vitreous body (Brückner 1943 a) probably finds readier access into the a.ch. in the absence of the crystalline lens. One drop of DFP may in this case not be sufficient to inactivate all the ChE in the aq.h. The remaining ChE would then restrain to some extent the increase of ACh and the total effect of DFP would thus be slightly depressed. However, even Dunphy's theory scarcely provides an explanation for the absence of congestion in aphakic glaucomatous eyes, for in the present experiments the congestive signs were clearly evident in the rabbits despite the presence of the crystalline lens, on the one hand, and the increased ChE activity of aq.h., on the other hand.

Leopold and Comroe (1946 b) observed that 1 per cent physostigmine and 0.1 per cent DFP produce a similar increase in capillary permeability. Assuming that the ChE activity of the aq.h. is directly proportional to the amount of aq.h. protein, the changes effected in the ChE activity by these concentrations of the two substances should at least have the same trend, and even be approximately equal in amount. Since in the author's investigation, even large dosages of physostigmine gave results that were opposite to those obtained with DFP, factors other than an increased protein content probably influence the final ChE activity of aq.h. However, increased proteins presumably play some part, as considerable foam was regularly seen in those samples in the author's experiments in which the ChE activity was found to be increased following the administration of polyalkylphosphates. Foam was also seen, it is

true, in a few samples showing inhibition of ChE in the physostigmine and neostigmine tests.

Siliato (1953) studied the effect of isonicotinic acid hydrazid on the ChE activity of guinea pig serum and aq.h. and found increased activity in both. He concluded that the test substance behaves like a sympathomimetic. This appears to be a logical conclusion if we proceed from the fact that parasympathomimetics inactivate ChE, *i.e.*, are AntiChE:s. The findings in the present studies of an increased ChE activity of the aq.h. after the administration of substances that produce a parasympathomimetic effect do not permit the acceptance of Siliato's conclusions without further evidence.

A study by Voss (1950) on the effect of Mintacol instilled into the rabbit eye after a lancet incision gave exceptional results. He found a significant decrease in the protein content of aq.h. in comparison to the control eye and acceleration of healing of the wound. He ascribed these effects to lowered capillary permeability following the increased permeability produced by Mintacol. The results obtained in the present investigation render it difficult to understand the decreased protein content of aq.h., since in this work Mintacol had the ChE-increasing effect of other polyalkylphosphates. In the author's opinion the copious foam formation in also these samples indicated an increased rather than a decreased protein content. On the other hand, promotion of the adhesion of the wound by an increased amount of fibrin is comprehensible.

The advantageous effect of AntiChE in glaucoma and the increase in capillary permeability are further evidence in support of the opinion of, among others, Christini (1949), Magitot (1951) and Sugar (1951) that the basic cause of glaucoma lies in the disturbed balance of the blood circulation system. The most important site of action of the entire mechanism is undoubtedly the blood—aq.h. barrier, changes in which affect the osmotic pressure and thus the entire fluid circulation of the eye, *i.e.*, the secretion and ultrafiltration mechanisms (Kinsey and Grant 1944). To obtain an advantageous AntiChE action and reduced intraocular pressure, the optimal frequency of instillations and amount of AntiChE should be worked out in order to prevent the sensitively changing balance in the eye from inclining beyond favourable limits. In the light of clinical practice and of these experiments it

appears that very large dosages, even if they sometimes may seem reasonable, are not to be recommended. Thus in clinical studies it has been found that the highest concentration of, for instance, Mintacol is 1: 6,000. In the present investigation already this concentration was found to increase the ChE activity of aq.h. Linn and Tomarelli (1952) considered 0.1 per cent DFP excessively powerful for routine use. The danger of employing a concentration of 1.0 per cent DFP, which also is reported to have been administered (Leopold and Comroe 1946 b, Gifford 1948, Böck and Veitl 1949), is therefore obvious. An excessively slight change in the permeability, again, may leave the effect below the threshold required for the lowering of intraocular tension.

An increase of ChE activity in aq.h. must therefore probably be regarded as an unfavourable symptom of excessive increase in capillary permeability. In addition to congestion, plasmoid aqueous undoubtedly also tends to obstruct the outflow of intraocular fluid and thus to increase intraocular tension. The fact that several different miotics continue to be in use is evidence that a definitive ideal medium has not yet been found.

SUMMARY

Part I of this investigation deals with determinations of the cholinesterase activity of aqueous humour in man, rabbit, cat, cow, horse, pig and sheep.

By biological methods using m. rectus abdominis of the frog and the dorsal muscle of the leech for the determination of the acetylcholine content it was possible to demonstrate that, almost without exception, the aqueous humour of the above mentioned mammals shows cholinesterase activity, although the amounts are small. The quantitative values are highly approximate. No essential differences between the values for the various species of animals were demonstrable.

Confirmation was obtained of the previously known fact that cholinesterase activity in secondary aqueous humour is greatly increased. In view of the limitations of the methods it was difficult to demonstrate definitely whether the cholinesterase was specific or non-specific, but certain results obtained seem to indicate that at least the greater part of the cholinesterase of the rabbit and the cat is non-specific.

Part II covers studies of the influence exerted on the cholinesterase activity of aqueous humour by the earlier known reversible anticholinesterases, *i.e.*, physostigmine and neostigmine, and by certain of the new anticholinesterases of the polyalkylphosphate group, *i.e.*, DFP, TEPP, HETP, Mintacol and Tabun. The latter have chiefly an irreversibly inhibitive action on cholinesterase.

This investigation demonstrated that physostigmine and neostigmine either inactivate the cholinesterase in aqueous humour or produce no demonstrable change. On the other hand, the cholinesterase activity of aqueous humour is frequently increased clearly by polyalkylphosphates even in the usual therapeutic concentra-

tions. In low concentrations they either produce no demonstrable change or have a slightly inactivating action.

The increase in cholinesterase activity is probably a result of increased capillary permeability, which is an already previously known effect of all anticholinesterases. This action appears to be very marked in polyalkylphosphates. The flow of proteins into the anterior chamber caused by the change in permeability presumably also conveys with it such a large amount of cholinesterase that the instilled anticholinesterase, even a powerful one, is not adequate for its inactivation.

A number of findings reported in the literature which conflict with the observations of the writer are discussed.

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